Rejuvenating the brain's endogenous regenerative potential: focus on the role of MANF in brain development and ischemic brain injury

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CONTENTS

ABSTRACT

Stroke is one of the leading causes of death and a major cause of disabilities in adults. More than half of stroke victims suffer some type of disability, ranging from different levels of minor weakness in a limb to a complete loss of mobility. Currently, treatment of stroke requires a stringent rehabilitation programs. Nevertheless, two thirds of all patients will still have some type of difficulty with regular daily activities. Recent experimental findings raise the possibility that functional improvement after stroke may be achieved through neuronal replacement by endogenous neural stem cells (NSCs) residing in the adult brain. Therefore, additional understanding of the properties of NSCs will help to identify their optimal potential in cell-based therapy. Neurotrophic factors are a family of proteins that are important in neuronal development and function, and have been studied as possible treatments for ischemic brain injury. In addition to Brain-Derived Neurotrophic Factor (BDNF) and Glial cell line-Derived Neurotrophic Factor (GDNF), Mescenphalic Astrocyte-Derived Neurotrophic Factor (MANF) and Cerebral Dopamine Neurotrophic Factor (CDNF), that form a distinct family of evolutionary conserved proteins with neuroprotective effects, have potential for the treatment of stroke. While MANF has been shown to protect cortical neurons from death in a rodent model of ischemic brain injury, the effects of post-stroke MANF administration on cellular processes during the recovery phase are poorly understood. To shed light on the possible regenerative potential of MANF for the injured brain, we need to first investigate the roles of endogenous MANF in neural stem cells (NSC) under a normal or pathological conditions. We developed and optimized a work platform for studying the regulation and effect of MANF on biological properties of NSCs and cortical development. Our findings reveal an important role of MANF in neurite outgrowth and neuronal migration in the developing cortex. In addition, we demonstrated that endogenous MANF has the potential to protect NSCs against oxygen and glucose-deprivation conditions. Next, using neurosphere and subventricular zone (SVZ) explant cultures, we further studied the effect of MANF administration on cell differentiation and migration. We present the data that exogenously added MANF can induce neural/glial differentiation and promote cell migration out of SVZ explants. Also, utilizing the advantage of NSCs as a target for MANF, we discovered that exogenous MANF can induce the phosphorylation of STAT3 in NSCs. Finally, we used the rat model of ischemic stroke to compare the effects of MANF and GDNF in neurogenesis after stroke. While injection of GDNF into lateral ventricle has a strong mitogenic effect to increase neurogenesis in SVZ, it does not induce migration of neuroblasts towards the ischemic area. In contrast, MANF facilitates the migration of neuroblasts towards the lesioned cortex. Regarding long-term infusions in the peri-infarct zone, both GDNF and MANF recruited the neuroblasts to the infarct area. However, only MANF accelerated functional recovery after stroke. In summary, this work has extended the knowledge of MANF's capacity for neuronal differentiation as well as migration, and the regenerative capacity for its therapeutic use in further studies.

ABBREVIATIONS

6-OHDA	6-hydroxydopamine
AAV	adeno-associated virus
AKT	Protein kinase B (PKB)
APC	adenomatous polyposis coli
BBB	blood brain barrier
Bcl-2	B-cell lymphoma 2
BDNF	brain derived neurotrophic factor
bFGF	basic fibrobasic growth factor
bHLH	basic helix-loop-helix
Bmi-1	B lymphoma Mo-MLV insertion region 1 homolog
BMP	bone morphogenetic protein
BP	basal progenitor
BrdU	5-Bromo-2´-Deoxyuridine
CBF	cerebral blood flow
CBP	CREB-binding protein
CDNF	cerebral dopamine neurotrophic factor
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CP	cerebral plate
CREB	cAMP response elememt-binding protein
CT-1	cardiotrophin-1
Ctip2	COUP-TF-interacting protein 2
DCC	deleted in colorectal cancer
Dcx	doublecortin
DG	dentate gyrus
ECM	extracellular matrix
EGF	epidermal growth factor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
Fezf2	FEZ family zinc finger protein 2
GABA	γ-aminobutyric acid
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GPCR	G-protein coupled receptor
IGF-1	insulin-like growth factor
JNK	Jun N-terminal kinase
KO	Knock-out littermates
LIF	leukemia inhibitory factor
LV	lentivirus

MANF	mescenphalic astrocyte-derived neurotrophic factor
MAP	microtubule-associated protein
MAPk	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
MMP	matric metalloproteinase
NEP	neuroepithelial
NF	neurofilament
NGF	nerve growth factor
Ngn1	Neurogenin1
Ngn2	Neurogenin2
NPC	neural progenitor cell
Nrg 1	neuregulin 1
NSC	neural stem cell
NTF	neurotrophic factor
OGD	oxygen-glucose deprivation
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
RB	retinoblastoma
RG	radial glial
RhoA	Ras homolog gene family, member A
RMS	rostral migratory stream
RTK	receptor tyrosine kinase
Satb2	Special AT-rich sequence-binding protein 2
SCA17	spinocerebellar ataxia 17
SDF-1	stromal cell-derived factor-1
SGZ	subgranular zone
SN	substantial nigra
SNpc	substantial nigra pars compacta
SOX	SRY-related high-mobility groups (HNG)-box protein
STAT	signal transducer and activator of transcription
SVZ	subventricular zone
Tbr	T-box protein
TGFα	transforming growth factor-α
TH	tyrosine hydroxylase
Trk	tyrosine kinase receptor
TTC	2,3,5-triphenyl-2H-tetrazolium chloride
TUNEL	Terminal deoxynucleotidyl transferase dUPT nick end labeling
UPR	unfolded protein response

VEGF	vascular endothelial growth factor
VZ	ventricular zone
WNT	Wingless type
WT	wild-type littermates

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications, herein referred by their Roman numerals (I-III):

(I) **Kuan-Yin Tseng**, Tatiana Danilova, Andrii Domanskyi, Mart Saarma, Maria Lindahl, Mikko Airavaara (2017) **MANF is essential for neurite extension and neuronal migration in developing cortex**. eNeuro, pii: ENEURO.0214-17.2017, DOI: 0.1523/ENEURO.0214-17.2017.

(II) **Kuan-Yin Tseng**, Jenni Anttila, Konstantin Khodosevich, Raimo Tuominen, M, Maria Lindoholm, Andrii Domanskyi, Mikko Airavaara (2017) **MANF promotes differentiation and migration of neural progenitor cells with potential neural regenerative effects in stroke**. Molecular therapy, pii: S1525-0016(17)30435-5, DOI: 10.1016/j.ymthe.2017.09.019.

(III) Seong-Jin Yu, **Kuan-Yin Tseng**, Hui Shen, Brandon K. Harvey, Mikko Airavaara, Yun Wang (2013) **Local Administration of AAV-BDNF to Subventricular Zone Induces Functional Recovery in Stroke Rats**. PLoS One 8(12): e81750

1 INTRODUCTION

Stroke is the one of leading cause of death, accounting for 10% of all deaths, and a major cause of disabilities in adult (Lopez et al., 2006). More than half of stroke victims suffer some type of disability, ranging from different levels of minor weakness in a limb, to a complete loss of mobility in one side of the body. In Finland, stroke mortality has been declining to 8.9% of all deaths and is surpassed by cancer (Meretoja et al., 2011). Despite this, stroke incidence is strongly age-dependent and Finland has one of the most rapidly aging populations in Europe, with 17.2% of the population currently aged above 65 years (Sivenius et al., 2009). If primary prevention does not improve, Finland faces a rapid growing number of stroke patients. Optimal treatment for stroke should be developed to satisfy future increases in demand and ensure the best functional recovery after stroke. Current clinical treatment strategies for stroke primarily focus on removing the embolic clot to rescue cells from dying. The therapy for stroke is limited by a narrow therapeutic time window after onset of stroke, and lack of effective remedies for days after stroke (Barkho and Zhao, 2011). Recent findings raise the possibility that functional improvement after stroke may be achieved through neural replacement by endogenous neural stem cells/neuronal progenitor cells (NSC/NPCs) residing in the adult brain, such as in the subventricular zone (SVZ). Using a filament-based middle cerebral artery occlusion (MCAO) model in adult rodents, many studies have shown that within the first week after a focal ischemic insult there is a pronounced loss of striatal and cortical neurons (Belayev et al., 1996; Kokaia and Lindvall, 2003). However, there is a major increase in NSC proliferation within SVZ indicating that ischemic injury increases endogenous regenerative processes. Increased NSC proliferation has also been seen in both the hippocampus and the SVZ after both trauma and seizures during the first week after injury, but the rates of proliferation return to normal after several weeks. In addition to increased number of NSC/NPCs in the SVZ, there is increased migration of NPCs from SVZ to damaged regions (Yamashita et al., 2006). The recruitment of NPCs to the lesioned site raises the possibility of new-generated neurons replacing apoptotic neurons (Barkho and Zhao, 2011). However, in ischemic cortical stroke, very few adult-born

neurons in the ischemic cortex have been found, suggesting that the injured cortex is a nonpermissive environment for neuronal differentiation, either because of lack of survival cues, neurodifferentiation signals, or the presence of signals inhibitory for neurogenesis (Arvidsson et al., 2002).

Neurotrophic factors (NTFs) are proteins important for neuronal survival, regeneration and remyelination. Nerve growth factor (NGF) was the first neurotrophic factor demonstrated to be required for normal neuronal development (Airaksinen and Saarma, 2002). Another effect of NGF is the induction of neurite outgrowth (Angelastro et al., 2003) and potential clinical applications of this property, are being investigated, for instance, the use of the dorsal root ganglion cell together with NGF for regeneration of spinal ganglion neurons (Hu et al., 2005). Brain-derived neurotrophic factor (BDNF) is essential for the production of neurons from hippocampal progenitors, being required during proliferation to trigger neuronal fate (Bull and Bartlett, 2005). Moreover, BDNF is implicated in the dentate gyrus in response to neurogenic stimulation by exercise and has a role in mediating the effect of antidepressant drugs on hippocampal neurogenesis (Jun et al., 2012). Furthermore, BDNF has been shown to reduce motoneuron death after axotomy in neonatal animals and in adults after ventral root avulsion (Kishino et al., 1997; Novikov et al., 1997). Intrathecal administration of BDNF reverses both soma atrophy and reduction of choline acetyl transferase in motoneurons. Glial cell line-derived neurotrophic factor (GDNF) is a potent survival factor for midbrain dopaminergic neurons and prevents motoneuron cell death following ventral root avulsion in adults (Matheson et al., 1997). It also has a chemoattractant effect on neuronal precursor cells in the rostral migratory stream (d'Anglemont de Tassigny et al., 2015). Evidence exists that GDNF might directly affect peripheral nerve regeneration. Thus, neurotrophic factors such as NGF and BDNF delivered through an intracerebral route could become useful in therapeutic regimens. It is also envisioned that genetic engineering of NSCs that synthesize certain neurotrophic factors before transplanting or migrating into lesioned sites may augment the efficacy of both NSCs and neurotrophic factors in clinical situations.

Other neurotrophic factors are less defined in the literature. Cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF), located at endoplasmic reticulum (ER) and secreted in response to ER stress, are proteins that constitute a novel, evolutionarily conserved neurotrophic factor family expressed in vertebrates and invertebrates (Lindholm et al., 2007; Lindahl et al., 2017). The purpose of the present studies was to characterize the role of MANF on NSCs and its effects in neuronal differentiation, migration, and cortical development. Moreover, the neuroregenerative potential of MANF in a rat model of cortical stroke was explored.

2 REVIEW OF THE LITERATURE

2.1 Defining neural stem cells and their properties

As the central nervous system (CNS) develops, neural stem cells (NSCs) generated from neuroepithelium produce more specified neural restricted precursor cells and glial restricted precursor cells (Temple, 2001). Undifferentiated neural precursor cells differentiate and migrate to appropriate locations in which cells undergo further maturation; neural/glial cells become postmitotic, and neuronal cells send projections to appropriate targets and make synapses (Filous and Silver, 2016). As development proceeds, the number of NSCs is much diminished and by birth these cells represent only a small fraction of dividing cells located in restricted regions of the brain. Coincident with this decrease, the number of progenitor cells increases gradually and more mature differentiated cells can be identified as they migrate from the proliferating zones to their terminal locations. Throughout the CNS, neurogenesis is followed by gliogenesis which is followed in turn by the development of neuronal connections and subsequent myelination of axons (Borrell and Reillo, 2012). These complex changes ultimately result in the formation of the adult brain which undergoes, under non-pathological conditions, little new neuronal augmentation, except for the hippocampus and olfactory bulb.

2.1.1 The cellular properties of multipotent neural stem cells

The neural stem cells have the cardinal properties of stem cells -- the ability to self-renew and to generate differentiated progeny (Anderson, 2001). During early mammalian embryogenesis, the newly specified neuroectodermal cells which make up the neural plate and neural tube are known as neuroepithlial (NEP) cells (Haubensak et al., 2004; Wilson and Houart, 2004; Dwyer et al., 2016). In vivo, NEP cells have been observed to undergo multiple rounds of symmetrical cell division for expansion of the progenitor pool. In addition, NEP cells can undergo asymmetrical division in which one NEP daughter cell and one nascent neuron are produced (Miyata et al., 2004; Kon et al., 2017). These first-born neurons then migrate away from the proliferative ventricular zone to the mantle zone. Shortly after the onset of neurogenesis, NEP cells begin to acquire a glial

identity, forming a new neural stem cell population such as radial glial cells (RG cells) (Howard et al., 2008; Kazanis et al., 2008). Initially, RG cells were identified as scaffolding for newly generated migratory neurons (Heng et al., 2010), but now they are thought to be the primary progenitors of most neurons throughout the CNS and also to give rise, via lineage-restricted intermediate precursors, to the two main glial cell types: astrocytes and oligodendrocytes (Gritti and Bonfanti, 2007). In the nervous system, there is a distinct progression of lineage differentiation whereby RG cells first give rise to neurons and later to astrocytes and oligodendrocytes (Martynoga et al., 2012). NEP and RG cells can be cultured in vitro as neurospheres in the presence of fibroblast growth factor (FGF2) as well as epidermal growth factor (EGF), and in cultures they are multipotent and exhibit a self-renewal capacity (Ciccolini and Svendsen, 1998; Tropepe et al., 1999; Yoon et al., 2004; Chen et al., 2015b).

Following the transition to the RG fate, some progenitor cells begin to divide asymmetrically to generate a RG cell and another differentiated daughter cell, which migrates away from the apical progenitor domain and begin neuronal differentiation (Martynoga et al., 2012). In the forebrain, the cell lineages that produce neurons are very complex, and there are fate-restricted progenitor stages between stem cells and postmitotic cells. These intermediate cells have been called basal progenitors (BPs). They are generated in the developing telencephalon by asymmetric division from RG cells (Haubensak et al., 2004). In the dorsal telencephalon, which gives rise to the cerebral cortex, BPs divide symmetrically to generate two neurons and are thought to generate most cortical projection neurons, including early-born neurons in deep cortical layers and late-born neurons in superficial cortical layers (Miyata et al., 2004; Farkas and Huttner, 2008; Jiang and Nardelli, 2016). Thus, BPs appear to be unipotent and incapable of self-renewal in vivo, however, their potential has not been conclusively analyzed *in vitro*.

It is now commonly accepted that the adult mammalian brain is not just a static postmitotic organ, but also contains populations of cells with stem cell potential, although each population is distinct in regard to its differentiation capacity (Kirby et al., 2015). Adult neural stem cells within the SVZ are descendants of RG cells which line the

embryonic lateral ventricle (Kriegstein and Alvarez-Buylla, 2009). In rodents as shown in Figure 1, the predominant role of the SVZ stem cells is to generate new neurons which tangentially migrate through the rostral migratory stream (RMS) to the olfactory bulb where they differentiate into interneurons (Sun et al., 2010). The adult SVZ harbors four classes of cells: Type A, B, C and ependymal cells (Duan et al., 2008; Gil-Perotin et al., 2009). Ependymal cells are relatively quiescent in vivo but retain the ability to enter the cell cycle and may respond to injury by proliferation (Kempermann et al., 2004). Type B cells undergo asymmetric cell division to generate a transient amplifying population of rapidly dividing SVZ cells (type C cells) that in turn generate migratory neuroblasts (type A cells) which migrate along the RMS to the olfactory bulb (Doetsch et al., 1999b; Doetsch et al., 1999a; Jessberger, 2016). In addition to the germinal zone of the SVZ, continued neurogenesis has been described in the granular layer of dentate gyrus (DG) (Kuhn et al., 1996; Horowitz and Villeda, 2017). Progenitor cells are found along a thin strip of cells, referred to as the subgranular zone (SGZ), between the hilar regions and the granular cell layer (Gage et al., 1998; Guo et al., 2012). It has been shown that these cells have diverse functions in memory, learning, and cell replacement (Veeraraghavalu and Sisodia, 2013; Amrein, 2015). Furthermore, ischemic insults have been demonstrated to trigger neurogenesis from neural stem or progenitor cells in the SVZ of the lateral ventricle and dentate gyrus (DG) of the hippocampus (Kokaia and Lindvall, 2003; Kojima et al., 2010). Although the transcriptional and cellular events that maintain NSC identity in adult brain remain elusive, evidence suggests that the underlying mechanisms probably share a common, early embryonic gene expression program.

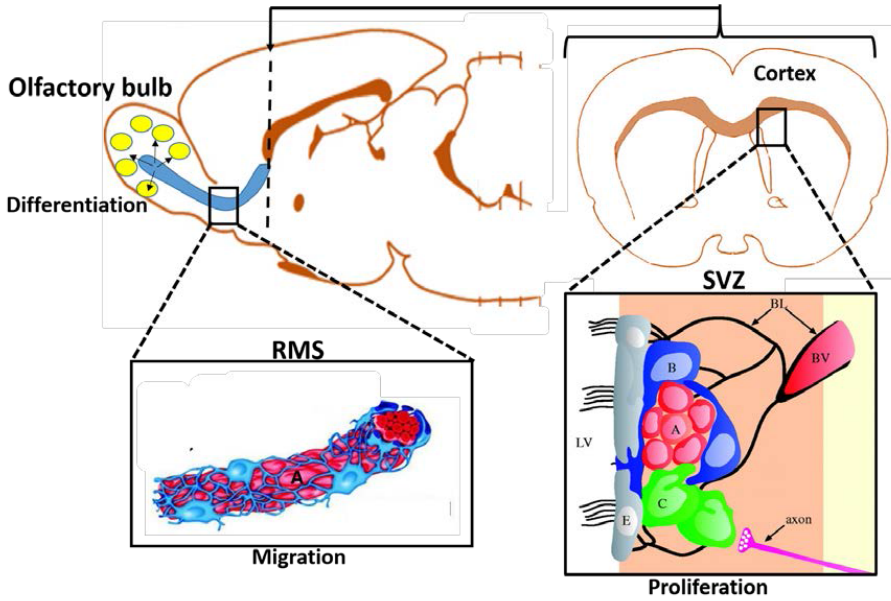

Figure 1. Schematic representation of the localization and cellular composition of the adult subventricular zone in the rodent brain. Neuroblasts generated in the SVZ niche migrate to the olfactory bulb and differentiate into granular and periglomerular GABAergic interneurons (yellow sphere). SVZ astrocytes in this region (B, blue) are stem cells which generate migrating neuroblasts (A, red) destined for the olfactory bulb via a rapidly dividing transit-amplifying cell (C, green). Region in box is expanded at right to show the relationship of cells in this region and some elements of the SVZ niche and RMS. Multi-ciliated ependymal cells (E, grey) line the walls of the lateral ventricle. Chains of neuroblasts (A cells) travel through tunnels formed by processes of SVZ astrocytes. Transit-amplifying cells are found in small clusters adjacent to the chains. Signals released from axons (pink) regulate proliferation and survival in this region. A specialized basal lamina (BL, black) extends from perivascular cells and contacts all cell types. Endothelial cells, blood vessels (BV) and the basal lamina are all likely key components of the niche. Modified from (Quinones-Hinojosa et al., 2006; Riquelme et al., 2008)

2.1.2 Self-renewal/proliferation of NSCs

Self-renewal and maintenance of NSCs in the SVZ and SGZ niches are mediated by attachment and various signals emanating from the endothelial cells of blood vessels and the specialized basal lamina (Williams and Lavik, 2009). β-catenin and cadherins play a role in maintenance of NSCs, and Wingless-Type (WNT) signaling overexpression of β-catenin results in exaggerated proliferation of NSCs (Nelson and Nusse, 2004; Holowacz et al., 2011). In addition, the polycomb family transcriptional repressor Bmi-1 is important for NSC self-renewal but not for NSC survival or differentiation (Molofsky et al., 2005; Corley and Kroll, 2015). Another approach to maintain NSC in an

undifferentiated state is to keep proneural proteins in an inactivating state. For example, the SRY-related high-mobility groups (HNG)-box protein-1, SOX1, SOX2, and SOX3 of B1 group proteins of SOX family participate in maintaining the NSC pool by blocking proneural basic helix-loop-helix (bHLH) protein activity (Corley and Kroll, 2015). Extracellular signals mediated by FGF2 and Notch1 are also crucial for maintaining the NSC pool and enhancing self-renewal within the SVZ niche (Hitoshi et al., 2004). Notch pathways are well established in promoting undifferentiated NSCs during developmental neurogenesis (Urban and Guillemot, 2014). *In vitro* self-renewal and proliferation can be meditated by high concentrations of mitogens, particularly EGF and FGF, in the culture medium (Hitoshi et al., 2004; Xu et al., 2017). In addition, ciliary neurotrophic factor (CNTF) augments self-renewal of neural precursors by promotion of Notch1 *in vitro*. The transforming growth factor (TGF)α, another ligand for the EGF receptor, may also have an influence on regulating proliferation of NSC/NPCs (Ahmed et al., 2009). Neurotransmitters as well as their receptors and transporters, like GABA and glutamate, directly regulate NSC/NPCs proliferation by changes in DNA synthesis and alternations in intracellular calcium concentrations (Dammerman and Kriegstein, 2000; Poluch and Juliano, 2015). These effects may be area-specific since the NPC response to glutamate and GABA is different between VZ and SVZ. Moreover, dopamine can have a direct effect on proliferation, since its D2 receptor, is expressed in NSC/NPCs (Baker et al., 2004; Sequerra, 2014). Moreover, hormones regulate NSC/NPC proliferation, since thyroid hormone increases NSC proliferation through its α-receptor and regulation of c-MYC expression (Lemkine et al., 2005). Furthermore, evidence indicates insulin-like growth factor 1 (IGF-1) promotes proliferation of NSCs in an estrogen-dependent manner (Perez-Martin et al., 2003; Sakthiswary and Raymond, 2012).

Cell cycle regulation is also essential for maintaining a proliferative state. Activation of the RAS/RAF/MEK/ERK protein kinase pathway shortens cell cycle length and thus alters proliferation rate (Orford and Scadden, 2008) . This pathway operates through P53, retinoblastoma tumor suppression (Rb), and E2F families of proteins. Rb has a role in promoting proliferation by meditating telomerase signaling in a cell type specific manner (Yoshikawa, 2000; Peng et al., 2012). The extracellular matrix is also important in

regulating NSC/NPC proliferation by directly affecting cell numbers or indirectly regulating the actions of growth factors. For example, integrins expressed by NSCs are activated by binding to extracellular matrix proteins which promote proliferation by activating intracellular phosphatidylinositol-3-kinase (PI3K) and AKT (Jacques et al., 1998; Christie and Turnley, 2012).

2.1.3 Survival of NSCs

The principle role of NSC apoptosis is to coordinate the number of proliferating cells, which will eventually affect brain size. Apoptosis is regulated by caspases and their telecncephalic transcripts are essentially linked with NSC survival. Caspase-3 and caspase-9 as promoters of apoptosis seem to be essential for early forebrain development in a region or cell population specific manner (Raff, 1998; Kuan et al., 2000; Ceccatelli et al., 2004). Staurosporine activation or caspase inhibitor-mediated blocking of caspase-3 leads to apoptosis or excess survival of NSCs, respectively, thus implying a caspase-dependent death pathway in NSCs. Apoptosis in NSCs via caspase activation can be blocked by activation of anti-apoptotic Bcl-2 (Hardwick and Soane, 2013). Another pathway that activates caspase-3 via Fas does not seem to cause apoptosis in NSCs. Antiapoptotic gene Bcl-X, which regulates neuron survival, is not involved with NSC apoptosis revealed by normal VZ after genetic manipulation. However, proapoptotic gene Bax, which is important for developmentally occurring neuronal death, seems to be important for naturally occurring apoptosis of adult NSCs through caspase and inositol 1,4,5-triphosphate (IP3) activation. Extrinsic factors are also important in preventing apoptosis since neuroepithelial NSC survival is mediated by FGF, EGF, insulin/IGF, and activation of antiapoptotic Bcl-2 in vitro (Chen et al., 2007; Huang et al., 2007). Factors such as c-Jun and N-terminal kinase (Jnk) seem to affect NSC survival in a regional and temporal manner, since Jnks decrease apoptosis in neuroepithelial NSCs and promote it in the forebrain VZ (Kuan et al., 1999; Yamaguchi and Miura, 2013). NTFs also play a role in NSC/NPC survival as shown by increases in apoptosis when the endogenous BDNF receptor, TrkB, and neurotrophin-3 receptor, TrkC, mediated signaling are blocked by antibodies (Barnabe-Heider and Miller, 2003; van Velthoven et al., 2014). This mechanism involves the downstream signaling target of Trks, PI3K and its adaptor

protein ShcA, suggesting an autocrine/paracrine action of neurotrophins in NSC/NPC survival (McFarland et al., 2006). Platelet-derived growth factor (PDGF) also acts through the PI3K pathway to promote survival of NPCs (Guillemot, 2007). The neurotransmitter glutamate promotes the survival of SVZ-derived NPCs (Brazel et al., 2005), and in contrast, hydrogen peroxide and rotenone induce apoptosis in NSC/NPCs (Lin et al., 2004; Wakai et al., 2014).

2.2 The processes of NSC differentiation

NSCs normally differentiate in multiple steps to produce the final cellular diversity in the mammalian CNS, starting from NSCs, through NPCs, to maturing terminally differentiated cell types including a vast variety of neurons, as well as astrocytes and oligodendrocytes. During cortical development, the generation of neurons and glia is temporally organized. Neocortical layer formation in particular occurs in a highly orchestrated manner. More recently, the discovery of genes that have layer and neuronal subtype specificity within the neocortex has made it possible to investigate the mechanisms underlying the specification of individual projection neuron subtypes. Finally, the onset of shaping of neuronal networks occurs during postnatal development and lasts to some extent throughout mammalian life.

2.2.1 The molecular mechanisms underlying neuronal differentiation

WNT signaling pathways play an important role in the NSC differentiation processes after the switch to support neuronal differentiation of NSC/NPCs instead of proliferation (Hirabayashi et al., 2004; Fernandez et al., 2016). WNT signaling is suggested to promote neurogenesis by directly activating proneural genes, Neurogenin1 and Neurogenin2 (Ngn1 and Ngn2) in clonal NPC cultures (Machon et al., 2003; Israsena et al., 2004; Jiang and Nardelli, 2016).

Proneural genes encode bHLH transcription factors, which have key roles in neurogenesis. These genes which are expressed in the mammalian telencephalon include Mash1, Ngn1, and Ngn2, and are also expressed in neocortical NPCs as well as in basal ganglia NPCs (Bertrand et al., 2002; Ross et al., 2003; Ghareghani et al., 2017). The most

important function for proneural genes is to direct NSC/NPCs toward a neuronal fate instead of an astroglial fate. Another function of these genes is to convert neuroblasts into mature neurons. *In vitro*, proneural genes have been shown to promote neuronal lineage of NPCs by direct transcriptional activation of downstream genes such as NeuroD (Nieto et al., 2001; Sun et al., 2001; Parras et al., 2004; Jiang and Nardelli, 2016). Moreover, Ngn proteins interact with histone acetylase CBP/P300 to activate target genes and, with a component of chromatin remolding complex, Brg1, to promote neurogenesis *in vitro* (Ge et al., 2006). Ngn1 and Ngn2 (Farah et al., 2000; Mizuguchi et al., 2001; Nakada et al., 2004; Cooper, 2013) are able to initiate neural differentiation and their sequential downstream targets include bHLH transcription factors NeuroD1, NeuroD2, Math2, Math3 and T-box proteins, Tbr1, Tbr2 (Hevner et al., 2001; Schuurmans et al., 2004; Englund et al., 2005). Ngn1 and Ngn2-mediated expression of Tbr1 and Tbr2 is restricted to cortical NPCs and neurons (Schuurmans et al., 2004; Dennis et al., 2016), which suggests that Tbr1 is essential for neural differentiation of some cortical NPCs and that Tbr2 is useful as a marker for intermediate cortical NPCs committed to glutamatergic fate. As opposed to Ngn1 and Ngn2, which are specifically involved in neural differentiation in the developing dorsal telencephalon, Mash1 is implicated in basal ganglia development (Jiang and Nardelli, 2016). Therefore, Ngn1 and Ngn2 are shown to control differentiation of cortical NPCs into a glutamatergic phenotype through activation of their target transcription factors. On the other hand, Mash1 seems to promote neural differentiation into a GABAergic phenotype through activation of Dlx homeodomain genes. Additionally, Ngn1/2 appears to repress Mash1 activation and activities in cortical NPCs, but Ngn2 alone seems to initiate a neocortical glutamatergic program independent of Mash1 repression.

2.2.2 Axonal formation, growth and branching

During neuronal differentiation, developing neurons are highly polarized and characterized by the heterogeneous compartmentalization of various cellular constituents into discrete and physiologically significant domains, the axon and dendrite (Lewis et al., 2013). Axonal outgrowth progresses through three stages: protrusion, engorgement, and consolidation (Dent et al., 2011). Protrusion is the extension of new membrane at the

edges of the growth cones, driven by filamentous actin (F-actin) polymerization. Engorgement occurs with microtubule-driven transport of membranous organelles and vesicles into an otherwise actin-dominated peripheral region. Consolidation results from changing a proximal growth cone into a cylindrical axon shaft, accompanied by the bidirectional movement of organelles and vesicles. As neuronal differentiation proceeds, axonal growth cone morphology changes dramatically as attractive and repulsive interactions between local microenvironmental cues and movement of underlying cytoplasmic axons toward appropriate synaptic targets come into play (Murray et al., 2010). Microtubule polymerization, heterodimers of two tubulin (α-tubulin and β-tubulin) in a polarized manner, is required to sustain axon elongation and branching (Lewis et al., 2013). Additionally, the disruption of microtubule-binding proteins such as APC (Shi et al., 2004), microtubule-associated proteins such as MAP1B (Takei et al., 2000; van de Willige et al., 2016) or proteins regulating microtubule reorganization are important. Mitogen-activated protein kinase (MAPK), which regulates microtubule stability and axonal transport through phosphorylation of Tau, is implicated in the growth and branching of axons (Mandelkow et al., 2004; Sayas et al., 2015). Following elongation of putative axonal neurites, neurofilaments (NF) are thought to provide structural support to mature axons via crosslinking of cytoskeletal elements mediated by the C-terminal region of high molecular weight NF subunits (Lee and Shea, 2014). This stabilization supports overall axonal neurite elaboration. Similarly, the interaction of NFs with each other and with other cytoskeletal elements is regulated by a complex hierarchy of kinase activities, including glycogen synthase kinase 3beta (GSK), and p42-44 MAPK (Lee and Shea, 2014). For example, BDNF, which promotes growth cone filopodial dynamics and axonal branching, activates MAPK (Meier et al., 2011). Moreover, p42-44 MAPK promotes NF axonal transport and induces NF axonal bundling (Lee and Shea, 2014).

Finally, upon reaching its target area, extensive axonal branching occurs during the formation of presynaptic contacts with specific postsynaptic partners. Axonal branches could arise either through the simple bifurcation of a growth cone tip, or through filopodial outgrowth from an axonal shaft to form collateral branches (Dent and Kalil, 2001). Neuronal activity can regulate branching through modification of the actin

cytoskeleton via RhoA activation, and local mRNA accumulates at presynaptic areas, indicating a correlation between local translation and synaptic activity (Bakos et al., 2015). Moreover, intracellular Ca^{2+} signaling has been shown to play a deterministic function in axon branching through activating the Ca^{2+}/calmodulin-dependent kinases (CAMKs) (Lewis et al., 2013). In addition to general growth-promoting factors, the candidates for axon guidance, such as ephrin, semaphorin, and netrin, act in an instructive manner which can actively attract or repel axons. When a growth cone encounters a guidance molecule, it extends away from chemorepellents and toward chemoattractants. For example, netrin can induce a high intracellular calcium concentration or a high cAM:cGMP ratio in the DCC receptor-expressed neurons and attracts growth cone turning to this guidance molecule (Chilton, 2006). In *in vivo* studies, EphrinAs have been demonstrated to pattern eye-specific projections to the appropriate layers of the lateral geniculate nucleus (LGN) in both mice and ferrets (Huberman et al., 2005). Conversely, the removal of ephrinA5 in knockout mice leads to temporal axons overshooting into posterior regions (Averaimo et al., 2016).

2.2.3 Dendritic branching morphogenesis

For most vertebrate neuronal populations, axonal morphogenesis occurs under electrically silent conditions or with low levels of spontaneous activity, whereas dendritic development proceeds in the context of various forms of neuronal activity (Acebes and Ferrus, 2000). Several factors participate in ensuring correct dendritic architecture, such as secreted factors, cell surface receptors, cell adhesion molecules, postsynaptic density proteins, signaling molecules, regulators of actin cytoskeleton, the molecules that control ER-Golgi protein trafficking, and transcription factors (Arikkath, 2012). For example, BDNF has been shown to be the most critical factor regulating dendrite outgrowth and branching through binding to the TrkB isoforms and activation of various signal pathways, such as the Ras/MAPK cascade, PI3 kinase/Akt pathway, and PLCγ-IP3-dependent calcium release (Harward et al., 2016). During neuron development, dendrites initially generate long, thin, filopodial protrusions but as differentiation proceeds, these labile dendritic filopodia are replaced first with polymorphic protrusions and then with knobby actin-rich spines containing postsynaptic density (PSD-95) clusters (Niell et al.,

2004; Chen et al., 2010). The molecular basis for cytoskeletal modifications underlying dendritic branching, similar to axonal branching, involves regulation of the synthesis and stabilization of actin and microtubule networks.

Recently, the role of unfolded protein response (UPR) signaling in dendritogenesis has been supported by experiments with cultured mouse hippocampal neurons. The UPR pathway plays a critical role in synthesis, folding and structural maturation of approximately one-third of all proteins produced in the cell, most of them being dedicated to secretion or membrane integration (Anelli and Sitia, 2008). UPR transduction involves the activation of ER membrane receptors that contain an ER luminal domain that senses the accumulation of misfolded proteins, including the inositol-requiring enzyme (Ire-1), the protein kinase (PKR)-like ER kinase (PERK), and the activating transcription factor 6 (Atf6) (Godin et al., 2016). The activation of the ER stress response is a pro-survival mechanism which expands the ER and reduces RNA translation to limit ER protein loading. The UPR also clears some misfolded proteins through activation of the ER-associated degradation (ERAD) process. However, when the UPR cannot cope with the overload of misfolded proteins then the cells activate a terminal UPR which finally leads to apoptosis (Walter and Ron, 2011). During neuronal development, dendrites acquire their morphology by considerable branch sprouting which comes with an increased need of protein production (Godin et al., 2016). Interestingly, in *Caenorhabditis elegans*, loss of ire-1 impairs dendritic morphogenesis in highly branched neurons (Wei et al., 2015). Moreover, BDNF-induced neurite outgrowth is strongly impaired in $Xbp1^{-/-}$ neurons (Hayashi et al., 2007). Since BDNF strongly enhances protein synthesis by promoting translation initiation in neurons, it could trigger the UPR and induce Xbp1 splicing in mouse hippocampal neurons. Subsequently, spliced Xbp1 is transported to the nucleus where it serves as a signal transducer for neurite outgrowth (Hayashi et al., 2007). However, the molecular targets downstream of Xbp1 signaling for neurite outgrowth remain to be elucidated. Indeed, downregulation of Hrd-1 level, one well-known ERAD-associated E3 ubiquitin ligase, counteracts the deleterious effect of mild ER stress on dendrite extension during RA-induced neuronal differentiation of P19 cells (Kawada et al., 2014). Altogether, these studies suggest UPR signals need to be temporally and

spatially fine-tuned during neuronal differentiation. Therefore, UPR levels are critical for neurite outgrowth which is vulnerable to both low and high signals.

2.3 Neocortex development

The CNS is made from cells that divide to form neuroepithelium, which folds into the fluid-filled neural tube. During the onset of neurogenesis, neuroepithelial cells divide asymmetrically in the VZ and SVZ of the anterior and dorsal neural tube to form radial glia, which produce the radially migrating newly born neurons of the neocortex (Tan and Shi, 2013). These neurons find their place in the six neocortical layers in an inside out fashion and mature to exhibit various neuronal types. GABAergic interneurons are generated mainly in subcortical regions and they migrate tangentially to the neocortex (Luhmann et al., 2015). The last steps of neocortical development are axonal outgrowth and synaptogenesis, which include making new connections and synapse pruning in an activity-dependent manner.

2.3.1 Neurogenesis in the developing cortex

There is a sophisticated signaling mechanism regulating the onset and maintenance of neurogenesis and gliogenesis (Figure 2). The onset of cortical neurogenesis and the transition from neuroepithelial cells to RG cells coincides with the onset of Notch signaling in the dorsal telencephalon as detected by the expression of the major Notch ligand Delta-like 1(Dll1) and the downstream transcription factors Hes1 and Hes5 (Martynoga et al., 2012). Notch is important for the instigation of RG development and for maintenance of neurogenic RG in an undifferentiated state. Moreover, Neuregulin 1 (Nrg 1) is expressed in the developing cortex and signals through its receptors ErB2 and ErB4 to promote RG identity and to suppress the differentiation of RG into astrocytes (Schmid et al., 2003; Sardi et al., 2006). Similar to the effect of enforced early activation of the Notch pathway, expression of a constitutively active form of a receptor for fibroblast growth factors also promotes precocious acquisition of RG cell identity (Yoon et al., 2004). Additionally, FGF2 maintains the proliferation of progenitors at the onset of neurogenesis by upregulating expression of cyclin D1 and down-regulating expression of the cyclin-dependent kinase inhibitor p27 (Lukaszewicz et al., 2002). Therefore, it

shortens the G1 phase of the cycle and decreases the proportion of neurogenic divisions. As neurogenesis progresses, RG cells would generate neurons directly or via basal progenitors indirectly. Pax6, Ngn1/2 and RA promotes the direct generation of neurons by RG cells. Meanwhile, several transcription factors (Ap2ɣ, Ngn2, Insm1, Tbr2) have been implicated in promoting the generation of basal progenitors from RG cells (Martynoga et al., 2012).

Other transcription factors and signaling molecules also promote the self-renewal of RGs (Wnt, Myc) and the proliferation of basal progenitors (Foxg1, Wnt/n-Myc) (Martynoga et al., 2012). Another important pathway with complex inputs into the regulation of cortical neurogenesis is the bone morphogenic protein (BMP) pathway. Administration of BMP4 to early cortical progenitors induces neurogenesis. However, later in cortical development, BMPs block neurogenesis and instead promote astrocyte differentiation (Mabie et al., 1999; Gamez et al., 2013; Scholze et al., 2014). Therefore, it is still largely unknown about how transcription factors interact with signaling pathways to regulate the generation and expansion of the different populations of neural progenitors. PDGF is suggested to promote neuronal fate in NPC cultures by binding to a tyrosine kinase receptor which in turn activates the intracellular SHP2-mitogen-activated-protein-kinase kinase (MEK)-ERK pathway that mediates neurogenic signals of a variety of growth factors (Katz et al., 2007).

Recently, compelling evidence has emerged to suggest that dynamic regulation of UPR signals governs the switch from direct to indirect neurogenesis (Laguesse et al., 2015). Abundant studies demonstrated that a progressive down-regulation of UPR in cortical progenitors acts as a physical signal to amplify BPs and promotes indirect neurogenesis (Paridaen and Huttner, 2014). The persistent elevation of the UPR resulting from defective protein translation and / or folding causes a decreased rate of indirect neurogenesis and subsequently a severe microcephaly. Accordingly, Grp78 mutant mice are also microcephalic (Mimura et al., 2008). As such, it is worth noting that only the Perk-eIF2α-Atf4 branch of the UPR is strengthened in mice deficient for the Elongator complex (Frank et al., 2010). This implies that Atf6 and Ire1 pathways only play minor

roles in this pathological process. Besides its contribution to the regulation of cortical neurogenesis, Atf4 also controls cell cycle progression of the earliest progenitors through regulation of Cyclin D promoter activity and migration of the earliest born neurons by unknown mechanisms (Frank et al., 2010). Altogether, those studies show that neuronal progenitors are acutely sensitive to Atf4 dosage and that a proper level of Atf4 is required for efficient neurogenesis in the developing mouse brain.

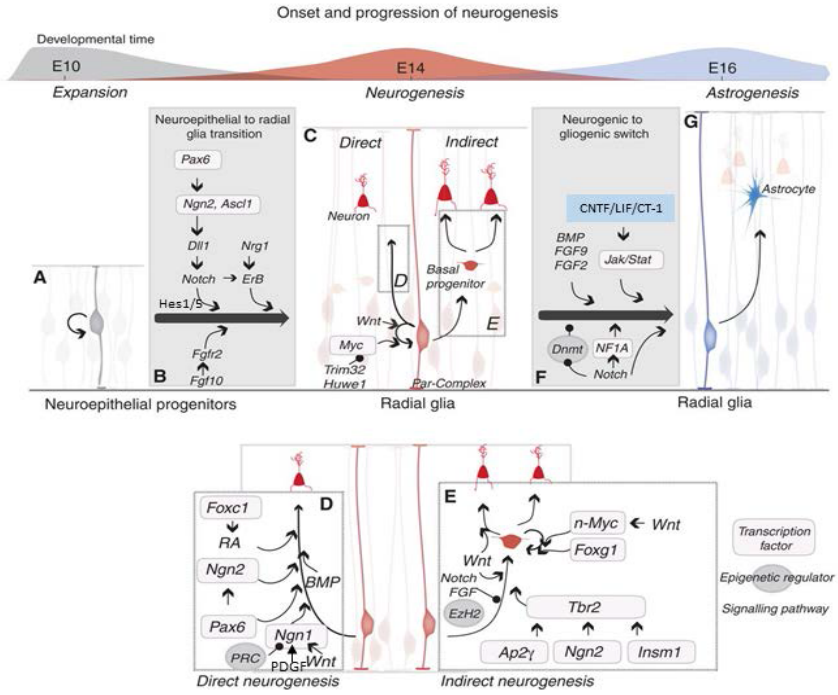

Figure 2. Induction of neurogenesis and gliogenesis in the embryonic neocortex. The onset of neurogenesis is concomitant with the transformation of neuroepithelial stem cells (A) into radial glial (RG) stem cells (C). Several signaling pathways, including the Dll1/Notch/Hes1/5, Nrg/ErB, and Fgf10/Fgfr2 pathways have been implicated in this transformation (B), and then RG cells generate neurons directly (D) or via basal progenitors (E). The pathways (Pax6/Ngn2, PDGF/Ngn1, Foxc1/RA) could promote the direct generation of neurons by RGs. Other transcription factors (Ap2γ, Ngn2, Insm1, Tbr2) have been shown to promote the generation of basal progenitors from RGs, whereas the Notch and FGF pathways and the epigenetic Ezh2 inhibit this step. Wnt, Myc et al. promote the self-renewal of RGs and the proliferation of basal progenitors (Foxg1, Wnt/n-Myc). The termination of neurogenesis results from the terminal differentiation of RGs into astrocytes (G). Multiple signaling pathways (Jack/Stat, Notch, BMP, FGF) synergize to elicit the neurogenic–to-gliogenic switch. The same pathways frequently operate in different temporal contexts to exert contrasting cellular effects. Adapted and reprinted by permission from Cold Spring Harbor Laboratory Press: Cold Spring Harb Perspect Biol, Martynoga, B et al., copyright 2012.

2.3.2 Gliogenesis: timing and control mechanisms

Around E18.5 in mouse cortical development, another major developmental transition occurs (Fig., 2). RG progenitors stop producing neurons, and the first astrocytes appear. During this process, most RG become responsive to BMP, EGF, or the cytokines CNTF, LIF, and cardiotrophin-1 (CT-1), and glial specific genes such as GFAP and S100B begin to be expressed (Guillemot, 2007). The competence of RG to respond to cytokine signals is achieved by the action of FGF2 and EGF (Lillien and Gulacsi, 2006). Most prominent among the pro-astrocytic signals is the cytokine-activated Jak/Stat pathway. Although Jak/Stat signaling is active at a low level in progenitors during neurogenesis and may even be required for RG identity, at the end of this period several mechanisms cause a sudden increase in Jak/Stat signaling. Firstly, several promoter elements in glial and Stat pathway genes are specifically methylated, which preclude Stat binding. This methylation is removed at the end of neurogenesis, in part, by Notch signaling (Takizawa et al., 2001; Martynoga et al., 2012). Similar to the cooperation between Notch and Jak/Stat signaling, there is pro-astrocytic synergy between BMP and LIF which is mediated by the formation of a complex between Stat3, Smad1, and the coactivator p300, which together strongly activate the GFAP promoter (Nakashima et al., 1999). Secondly, BMP and Notch signaling favor gliogenesis primarily by blocking the action of proneural genes, Ngn1 and Ngn2, which are potent antagonists of the Jak/Stat pathway (Sun et al., 2001; Scholze et al., 2014). Thirdly, the cytokine CT-1 starts to be expressed at high levels by differentiating neurons and this molecule appears to be the main activating ligand for the Jak/Stat pathway in vivo (Miller and Gauthier, 2007). The end results of these events is an increase in Stat activity, which become even more amplified because the Stat factors start to positively autoregulate their own transcription (Martynoga et al., 2012).

2.3.3 Neuronal subtype specification in the developing cortex

A number of layer-specific genes in the mouse neocortex have been identified (Fig., 3). As early as E11.5, when deep layer neurons are generated, Fezf2 expression in VZ cells may promote the expression of Ctip2, a zinc finger transcription factor in young neurons which, together with other genes, confers a subcortical projection neuron fate during

differentiation (Leone et al., 2008). At the same time, Fezf2 appears to repress Satb2 thereby repressing callosal identity. At later times during corticogenesis, when upper layer neurons are generated, Fezf2 expression is absent which relieves the repression of Satb2 (Leone et al., 2015). This, in turn, enables Satb2 to actively repress Ctip2 expression and promote the adoption of a callosal or corticocortical neuron identity. Although Satb2 and Ctip2 are coexpressed by individual neurons within layer V during a brief time window, the coexpression quickly resolves and Ctip2 is specifically stained in corticospinal motoneurons (CSMN) of layer V (Arlotta et al., 2005). Finally, it is clear that Tbr1 plays an important role in cortical development, particularly in regulating the differentiation of subplate neurons. Moreover, preplate gene expression during early corticogenenesis and layer VI neurons (corticothalamic neurons) are characterized by high levels of Tbr1 expression (Bulfone et al., 1999; Zhang and Jiao, 2015). Indeed, Tbr1 knockouts exhibit a variety of axon projection defects, with corticothalamic projections growing only as far as the internal capsule, callosal projections mostly terminating in the Probst bundle not crossing the midline, and thalamocortical projections reaching the internal capsule, but then turning away from the cortex (Hevner et al., 2001; McKenna et al., 2011).

At a later embryogenic stage, when upper layer neurons are generated, Cux1 and Cux2 are most expressed in corticocortical projection neurons of layer II-IV (Iulianella et al., 2003). The homeodomain genes Cux1 and Cux2 are initially expressed in SVZ cells and in their progeny in layers II-IV. The analysis of Cux2 knockout animals has revealed that Cux2 promotes the exit of SVZ cells from the cell cycle (Zimmer et al., 2004). Birthdating experiments suggest that Cux2-deficient SVZ progenitors repeat the cell cycle at higher frequencies than in wild-type controls, leading to an increase in the size of the SVZ progenitor pool (Cubelos et al., 2008). In addition, Cux1 and Cux2 are intrinsic and complementary regulators of dendrite branching, spine development and synapse formation in layer II-III neurons of cortex (Cubelos et al., 2010). As more evidence accumulates regarding the functional roles played by many subtype-specific genes, more understanding of the programs of gene expression that direct neuronal subtype differentiation in the neocortex should emerge (Zhang and Jiao, 2015).

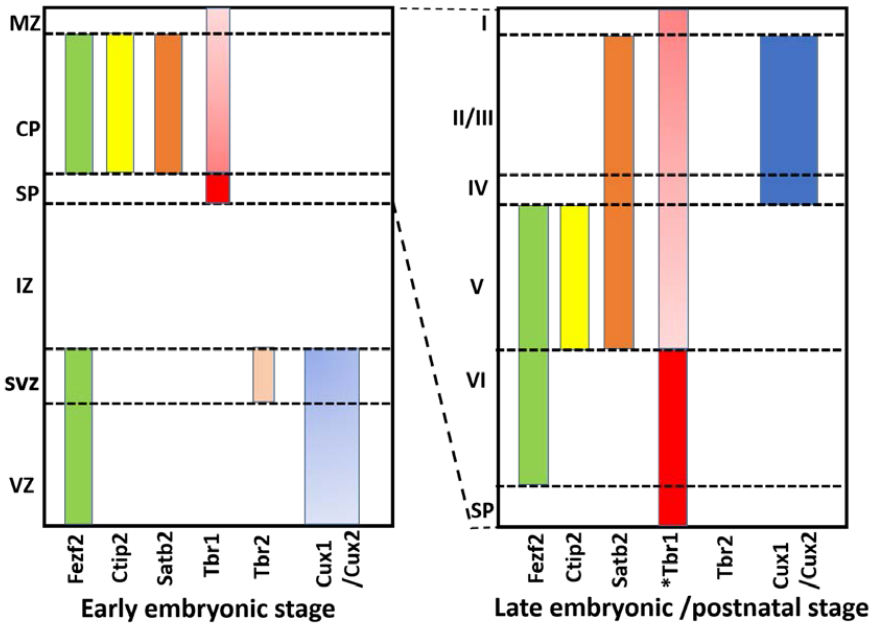

Figure 3. Gene expression patterns in the developing cerebral cortex during embryogenesis and early postnatal life. Summary of the expression patterns of the zinc-finger transcription factors, Fezf2 and Ctip2, the chromatin remodeling protein, Satb2, T-box transcription factor, Tbr1, and cut-like transcription factors Cux1 and Cux2 during embryonic and early postnatal development in the mouse. Dark and light red or blue indicate higher and lower relative levels of expression, respectively. A gene for which laminar or subtype expression varies by area within the neocortex is indicated by an asterisk. Abbreviations: MZ, marginal zone; CP, cortical plate; SP, subplate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Modified from (Leone et al., 2008; Martinez-Cerdeno et al., 2016).

2.3.4 Neuronal migration in the developing cortex

In the developing cortex, the migration of newborn cells is directed away from the proliferative zones, including the VZ and the SVZ. Radial migration is the main mechanism that creates the layered structure of excitatory neurons in the neocortex. Meanwhile, the interneurons migrate from subpallium to pallium in a tangential manner (Marin and Rubenstein, 2003; Dennis et al., 2016).

The successful execution of migration depends on the capacity of the cell to: (i) establish polarity; (ii) undergo directed migration to its final destination; and (iii) terminally differentiate to transmit and receive information (Heng et al., 2010). Neuronal

polarization includes 3 stages. The first stage is disruption of neuron symmetry resulting from the inhibition of the RhoA-ROCK-profilin IIa signaling pathway in submembranous domains, leading to destabilization of actin cytoskeleton, which in turn enables initial neurite sprouting (Da Silva et al., 2003; Konietzny et al., 2017). Other molecular pathways that regulate microtubule stability in neurons also influence neuronal polarity. Among them, the GSK-3 pathway controls cell polarity by regulating the microtubule affinity of various microtubule-associated proteins (MAPs) (Barnes and Polleux, 2009). The enlargement of the growth cone of presumptive axons followed by axon specification and elongation occur in stage 2 and stage 3.

When cells undergo migration, they utilize three mechanisms to arrive to their final positions: (i) somal translocation, whereby neuron cell body travels with pial contacting radial processes; (ii) locomotion, a process by which newborn neurons develop leading and trailing processes and migrate along RG fibers and; (iii) multipolar migration (Noctor et al., 2004; Subramanian et al., 2017). In addition, their migration is prefigured by activities of key proteins which interact with a host of other molecules to modify the actin and microtubule cytoskeletons (Heng et al., 2010). Central to the regulation of the actin cytoskeleton in neurons are several molecules, such as Arp2/3 (actin-related protein 2/3) complex (Goley and Welch, 2006; Pak et al., 2008), the RhoA-ROCK-LIMK-Cofilin pathway (Ge et al., 2006) and members of the ENA-VASP family of actin-binding proteins, which control the elongation of unbranched actin filaments (Goh et al., 2002; Kwiatkowski et al., 2007). The microtubule cytoskeleton and associated proteins are also important for cell migration and neuron differentiation. Microtubule filaments can be assembled from eight different α subunits, which heterodimerize with nine possible β subunits (Dutcher, 2001; van de Willige et al., 2016). Microtubule composition and its function are predetermined through different layers of molecular regulation: (i) gene expression; (ii) microtubule heterodimer composition; and (iii) post-translational modification. For example, studies of the genetic causes of lissencephaly and polymigrogyria in humans have identified tubulin-alpha1 (TUBRA1) (Keays et al., 2007) and tubulin-beta2b (TUBB2B) (Jaglin et al., 2009) to be crucial for cortical neuron migration and brain development. Moreover, a host of MAPs, like Map1b, Map2 and

Tau, have crucial functions in regulating cortical neuronal migration and axon outgrowth (Dehmelt and Halpain, 2004). Studies of several nonclassical MAP2s, such as doublecortin (Dcx), which binds to microtubules to increase their stability (Moores et al., 2004; van de Willige et al., 2016), are shown to be important, and loss of Dcx results in migration deficits (Bai et al., 2003). These observations together indicate that spatiotemporal availability of actin and microtubule cytoskeletal proteins, as well as their associated proteins with downstream effectors, operate within specific steps in the migration of newborn projection neurons to establish polarity and correct morphology, as well as the appropriate mode of cell migration. Furthermore, it is known that neuronal migration and neurite outgrowth are correlated and can depend on similar molecular mechanisms.

As the neuronal migration is implicated in corticogenesis, radial migration of newborn neocortical neurons has been well studied and exhibits four distinct phases. The first phase, after their generation at the ventricular surface, is a rapid radial migration to the SVZ. The second phase consists of migratory arrest for 24 hours or more, followed by a third phase of retrograde migration toward the ventricle and a final phase of polarity reversal and migration toward the cortical plates. During their third phase of radial migration, neocortical neurons acquire a transient multipolar phenotype (Tan and Shi, 2013).

Tangential migration is a mode of neuronal migration that is not associated with radial glial-guidance or direction in the CNS, although its cell mobility is similar to the steps of radial migration (Toma and Hanashima, 2015). Tangential migrating cells can acquire diverse morphological appearances with short compact processes, long elongated processes, or branched processes (Marin et al., 2006). Although tangential migration is also found throughout developing brains, it only persists in the RMS of adult brains, where migrating neuroblasts travel long distances through glial tunnels formed by astrocytes from the SVZ to the olfactory bulb. After variable subtypes of neurons arrive to their destined layers of neocortex, synaptic connections are established and

strengthened mainly in an activity-dependent fashion. The process is very complex and new knowledge is emerging frequently and will not be discussed further in this thesis.

2.4 Endogenous neural stem cell response to ischemic stroke

The potential of NSCs/NPCs to replace the loss of neurons and restore function after ischemic stroke has been shon during the last decade. Both endogenous and grafted NSCs/NPCs are known to have the ability to migrate to lesioned sites after brain injury and differentiate into new neurons (Barkho and Zhao, 2011). Several chemokines and growth factors have been shown to stimulate the proliferation, differentiation, and migration of NSCs/NPCs, and investigators have now begun to identify the critical downstream effectors and signaling mechanisms that regulate these processes (Table 1). Understanding the regulation of NSCs/NPCs properties and molecular pathways involved in stem cell homing into ischemic areas is vital for the development of new treatments. To ensure the best functional recovery, regeneration therapy may require the application of a combined approach that incudes cell replacement, trophic support, and neural protection.

Table 1. studies implicating the role of the growth factors and cytokines											
Molecule		FGF	EGF	VEGF	BDNF	GDNF	SDF-1α	*TGF-β*	TNF-α	IL-1	IL-6
Model		*In vitro, in vivo*	EGF knock-out mice	*In vitro,* transgenic mice, overexpression, intraventricular infusion	*In vitro,* BDNF conditional knock-out mice, intraventricular infusion, stroke model	*In vitro, in vivo,* intracerebral infusions in stroke model	*In vitro, in vivo*	stroke lesions	*In vitro, in vivo*	*In vitro, in vivo,* stroke models	*In vitro, in vivo*
Brain region		SGZ; SVZ	SVZ	SVZ	SVZ	SVZ; RMS	SVZ	SGZ; SVZ	SVZ	SGZ; SVZ	SGZ, SVZ
Effect of NSCs/NPCs (↑ indicates an increase in the respective variable, NE indicates that the signaling molecule had no effect on the respective variable, and ↓ indicates a decrease in the respective variable)	Proliferation	↑	↑	↑	↑/↓	↑	↑	↑	↑/↓	↑(antagonist)	
	Differentiation	NE			↑	↑		↑/↓	↑/NE/↓		↑ (astrogenesis)
	Apoptosis		↓	↓					↑/↓	↑	
	Migration			↑	↑	↑	↑		↑	↑(antagonist)	
	Survival				↑						↑/↓
	Reference	(Gritti et al., 1996; Matsuoka et al., 2003)	(Doetsch et al., 2002; Baldauf and Reymann, 2005)	(Jin et al., 2002; Wang et al., 2007; Mani et al., 2010)	(Ahmed et al., 1995; Benraiss et al., 2001; Chen et al., 2005; Schabitz et al., 2007; Grade et al., 2013)	(Kobayashi et al., 2006; Paratcha et al., 2006; Shang et al., 2011)	(Thored et al., 2006; Xu et al., 2007; Kokovay et al., 2010; Ramos-Cejudo et al., 2012)	(Zhu et al., 2000)	(Buttini et al., 1996; Kang et al., 2013)	(Guadagno et al., 2015; Pradillo et al., 2017)	(Suzuki et al., 2009)

2.4.1 The pathophysiological mechanism of ischemic stroke

Acute ischemic stroke is caused by cerebral artery occlusion through the loss or the reduction of cerebral blood flow, leading to an infarction of brain tissues. During the initial phase of the infarct, the loss of oxygen or glucose to the brain region results in depletion of intracellular ATP levels, causing membrane depolarization and blockage of protein synthesis (Barkho and Zhao, 2011). A major cause of neuronal death by oxygen and glucose depletion is through glutamate excitotoxicity, which can result from impaired ion exchange pumps, triggering the reversed extracellular release of glutamate by neurotransmitter transporters (Dirnagl et al., 1999; Kim et al., 2017). High concentrations of extracellular glutamate act on receptors on post-synaptic neurons and can lead to calcium influx, failure of mitochondria, energy depletion, and eventually neuronal death through apoptosis (Trendelenburg, 2014) . The area where the cells lose metabolic activities and quickly die in the absence of reperfusion is defined as the ischemic core. In most cases, the ischemic core is considered "out of reach" for neuroprotective treatments (Saver et al., 2010). The surrounding area, called the penumbra, is supplied partly by collateral blood vessels. Since it experiences a milder loss of cerebral blood flow (CBF, a 60-80% reduction), cells of the penumbra are initially able to prevent complete loss of ATP (Yan et al., 2015). However, some of the damage done to the brain following stroke comes from delayed effects, including release of nitric oxide, oxygen free radicals, and depolarizing waves of spreading depression originating from the ischemic core. Cells of the penumbra are exposed to these conditions under which they are unable to cope with increasingly harmful insults and the penumbra eventually converts to an irreversibly damaged ischemic core.

Besides the harmful effects on neurons, ischemia can damage the integrity of the neurovascular network via release of matrix metalloproteinases (MMPs) and other proteases secreted by endothelial cells that compromise the protective blood brain barrier (BBB) (Lo et al., 2003). The loss of neurovascular structural integrity results in a breakdown of the tight junctions between astrocytes and endothelial cells, which contributes to cerebral edema. The events described above are important factors to

consider not only when treating stroke patients, but also critical for cell-based therapies using endogenous or transplanted stem cells.

2.4.2 Stroke-induced neurogenesis

Using an embolic middle cerebral artery occlusion (MCAO) model in adult rodents, many studies have shown that within the first week after focal ischemic insult there is a major increase in NSC proliferation within the SVZ (Barkho and Zhao, 2011). Increased NSC proliferation has also been seen in both the hippocampus and the SVZ after trauma, seizures, and global ischemia during the first week after injury, and the rates of proliferation return to normal after several weeks (Parent et al., 2002; Kernie and Parent, 2010). It is well known that FGF-2 appears to play a critical role in regulating injury-induced NSC proliferation and this effect is attenuated in FGF-2 knockout mice *in vivo* (Addington et al., 2015). Much like FGF, SVZ proliferation after stroke was found to increase in vascular endothelial growth factor (VEGF)-overexpressing transgenic mice, suggesting VEGF may also contribute to NSC proliferation within the SVZ after injury (Thau-Zuchman et al., 2010). At two weeks after the injury, newly generated SVZ-derived cells re-route from the SVZ and RMS into the damaged area (up to 2mm distance), where some of these cells are found to express differentiated cell markers at later time points (Kokaia and Lindvall, 2003; Lindvall et al., 2004). Ultimately, the increased number of migrating neuroblasts found in the SVZ or dentate granule layer, the majority of which are neuroblasts predisposed to migrate to damaged regions, will not survive (Takasawa et al., 2002; Tonchev et al., 2005). Therefore, injury-induced neurogenesis is suspected of being regulated by extrinsic factors secreted from reactive cells within the infarct regions. However, the number of neurons generated from endogenous NPCs is extremely low (~0.2% of the striatal cells lost), and the survival of these new neurons in the lesioned area is minimal (Arvidsson et al., 2002). A major problem with stem cell migration to the injured stroke area is subsequent apoptosis of the newly migrated NSC. Recently, an approach to prevent this is characterized by the use of regulatory factors that have been implicated in neurogenesis, such as GDNF, BDNF, VEGF, granulocyte colony-stimulating factor (G-CSF), FGF–2, insulin-like growth factor–1, bone morphogenetic protein–7, epidermal growth factor, and transforming

growth factor–α (Azad et al., 2016) in order to protect the NSCs/NPCs. Alternative strategies to increase NPC proliferation include using anti-inflammatory drugs, noncoding RNAs, and hormones such as erythropoietin and growth hormone (Wang et al., 2004; Hoehn et al., 2005; Schouten et al., 2012). A complementary approach strives to limit NPC death through administration of G-CSF and insulin-like growth factor-1 to alter key survival pathways (Lee et al., 2006). Inhibition of P53 and the use of cyclosporine have also been studied as strategies to extend NPC survival (Luo et al., 2009; Erlandsson et al., 2011).

2.4.3 The migration of neural progenitor cells after brain injury

In recent years, interest in understanding the physiological and pathological processes of progenitor cell migration has increased, due largely to the discovery of neural progenitor cell migration in the adult brain under normal or injured conditions. Above, we have reviewed the mechanisms of neuronal migration in developing brains. In adult brains, migrating neuroblasts travel long distances through a glial tunnel formed by astrocytes from the SVZ to the olfactory bulb. Upon arrival at the olfactory bulb, neuroblasts switch to radial migration to reach their destination. Interestingly, under pathological conditions, these cells also have the capacity to adapt similar migration patterns in response to changes in their surrounding environment (Goings et al., 2004).

Upon neuroinflammatory injury, which occurs in stroke or trauma, chemokines are produced by reactive cells, such as astrocytes, and endothelial cells express higher levels of chemokines, like stromal cell-derived factor 1(SDF-1α) and VEGF. These injury-induced chemokines are reported to attract inflammatory cells and cause cell death in diseased or injured regions; however, chemokines can also direct neuronal progenitor cells (NPCs) to re-route towards a stroke-induced injury (Wiltrout et al., 2007). Chemokine-induced cell migration requires the remolding of the extracellular matrix (ECM). The chemotaxic functions of SDF-1 (Thored et al., 2006) and VEGF (Zhang et al., 2002) are known to be mediated by the activation of MMPs, which are a family of enzymes responsible for degrading the components of the ECM. For example, after ischemic injury, neuroblasts are in close proximity to the endothelial cells of the

vasculature and secrete MMPs to degrade laminin, one of the basement membrane proteins of the ECM, to mediate cell migration (Lee et al., 2006; Wang et al., 2006). Furthermore, it has been shown that MMPs, with their ability to disrupt the interaction between integrins expressed on the cell surface and the ECM, can activate cell surface molecules such as receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and membrane tetraspanin family of proteins (Barkho and Zhao, 2011). When these receptors are directly or indirectly activated they transduce signals through several pathways, including focal adhesion kinase, the Src family kinase Fyn, MAPK, SH2-SH3 adaptors, Rho-family GTPases, and phospholipid mediators (Barkho and Zhao, 2011). The activation of these signaling cascades ultimately results in a number of changes in plasma membrane localization, microtubule assembly, and interaction with the cytoskeleton, which are also implicated in NPC differentiation. Therefore, it has been suggested that the stroke-induced NPC migration and neuronal differentiation, including neurite outgrowth, may be correlated and depend on particular molecular mechanisms that can modulate both activities.

Additionally, stroke increases the expression of BDNF and other growth factor or NTFs in adult rat cortex or neostriatum near the rostral SVZ or RMS (Bath and Lee, 2010). In some instances, increased expression is sustained for up to 14 days, similar to the temporal pattern of ischemia-induced neurogenesis and neuroblast migration. However, the functions of these endogenous growth factors have been mostly implicated in NSC proliferation, neuronal differentiation, and survival after brain injuries.

2.4.4 Therapeutic application of endogenous NPCs

Since short-term pharmaceutical treatments for brain injury have had limited success, stem cell-based therapies are now the subject of intense investigation. Endogenous adult NSCs residing in the neurogenic niche may be beneficial for brain repair as a result of their ability to support neurogenesis and gliogenesis during adulthood. However, the microenvironment surrounding the NSCs changes drastically after brain injuries due to the reactivity of neighboring cells, such as astrocytes, endothelial cells, and microglia (Barkho and Zhao, 2011). The release of cytokines from these cells changes the

microenvironment and therefore the niche for endogenous adult NSCs. As mentioned above, adult NSCs in the SVZ initially experience a massive proliferative response within the first week of ischemic stroke in response to an increase of cytokines, like TNF-α (Katakowski et al., 2007; Yan et al., 2015). However, the majority of these cells either fail to survive or to differentiate into glial cells when stimulated by the high concentrations of inflammatory cytokines secreted by neighboring cells.

Even though endogenous adult NPCs have the capacity to replace lost neurons in animal models of cerebral ischemia, the potential for functional recovery in humans remains uncertain. In the rodent stroke model, the efficiency of endogenous adult NPCs at generating new neurons that will survive and repair the damaged area is extremely low (Kokaia and Lindvall, 2003). Therefore, one current strategy focuses on promoting NPC recruitment to the injured area using chemokines and growth factors within the lesioned area. These factors can attract large numbers of endogenous NPCs to the injured area, and some of these cells may participate in brain regeneration. Growth factors, such as BDNF (Benraiss et al., 2001; Vilar and Mira, 2016) and VEGF (Jin et al., 2002; Ottoboni et al., 2017), have been used with some success; however, functional recovery was limited. Other peptides that provide neuroprotection and recovery in ischemic stroke preclinical models include CART (Luo et al., 2013) and sonic hedgehog agonists (Jin et al., 2015; Jin et al., 2017) . More recently, it has been suggested that a combination of these factors might have a synergic effect to promote endogenous stem cell therapy and improve brain function. For example, simultaneous promotion of neurogenesis and angiogenesis could result in a better therapeutic outcome. However, increased angiogenesis can also lead to greater permeability of the BBB, causing more cerebral edema after the acute stage of a stroke insult (Zhang et al., 2000; Zhang et al., 2017). Thus, it is also important to define an optimal time period and specific dosage for any growth factor or cytokine treatment.

Therefore, modulating endogenous NSC/NPCs for brain repair is a critical issue facing stem cell therapy. Acquiring this knowledge will require more research into the basic biology of NSC/NPCs and their interaction with the surrounding microenvironment after brain injury.

2.5 Neurotrophic factors and ischemic brain injury

2.5.1 Classification of neurotrophic factors

NTFs are secreted proteins that produce their trophic effects by activating specific receptors on the neuronal cell surface. Trophic factors in the CNS are grouped into families based on structural homology, receptors, and common signal transduction pathways (Fig., 4). Four major classes comprise the family of NTFs: (i) neurotrophins include NGF, BDNF, neurotrophin-3 (NT-3) and -4 (NT-4); (ii) the GDNF family of ligands (GFLs) including neurturin (NRTN), artemin (ARTN) and persephin (PSPN); (iii) neurotrophic cytokines (neurokines) with CNTF, CT-1, CT-2, IL-6; and (iv) the newest family consisting of cerebral dopamine neurotrophic factor (CDNF) and mesencephalic astrocyte-derived neurotrophic factor (MANF) (Lindholm and Saarma, 2010).

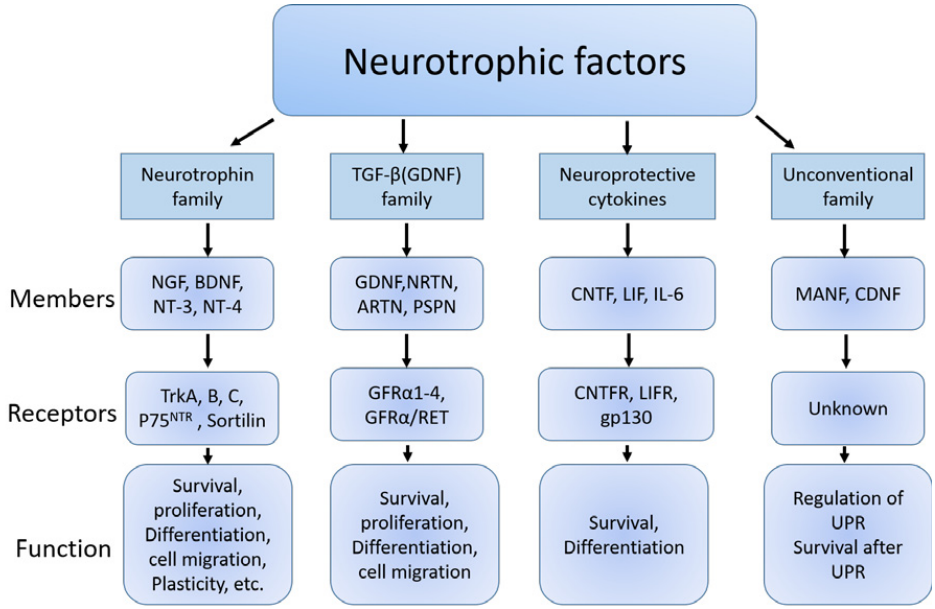

Figure 4. Classification of neurotrophic factors. Neurotrophic factors are classified into four groups depending on the structure and function. Neurotrophins function via binding to TrkB or $P75^{NTR}$ receptor in a variety of brain functions. Sortilin is newy identified proneurotrophin receptor mainly expressed in TGN and is shown to be involved in the processing for mature neurotrophins. The TGF-β family, also called as

GDNF family in brain. The molecules bind to GFRα, or GFRα-1-RET to function and function as a dimer. Neuroprotective cytokines consist of CNTF, LIF, and IL-6. Homodimers of these molecules bind to CNTFR, LIFR, or gp130, and receptor dimer formation is important for signal transduction. The mechanism of CDNF and MANF cytoprotective action is still largely unclear, and their ability to bind transmembrane receptors has not been demonstrated. Although MAND and CDNF can be secreted from cells, they are largely retained intracellularly in the ER for protein homeostasis. Modified from (Lindahl et al., 2017; Sato, 2017).

The actions of neurotrophins are mediated through the Trk family of RTKs (TrkA, TrkB, and TrkC) and p75 neurotrophin receptor (p75NTR), all of which are transmembrance receptors (Huang and Reichardt, 2001). Upon ligand binding, Trk receptors can activate such intracellular pathways as MAPK-pathway, PI3K-pathway and PLC-γ pathway; meanwhile p75 activates NF-kB and JAK-pathway. The p75NTR has high affinity for pro-neurotrophin, whereas mature neurotrophins have greater affinities for TrkB. Indeed, p75NTR, a death domain receptor, associated with Trk co-receptor enhances or suppresses neurotrophin-mediated cell survival (Bachis et al., 2012).

Similar to the neurotrophin family, the GDNF family of ligands are synthesized as precursor proteins that are cleaved into mature proteins. The members of the GDNF family are distant members of the TGF-β superfamily with low sequence similarity, but with identical spacing of seven conserved cysteine residues, and also belonging to cysteine knot proteins (Airaksinen and Saarma, 2002). The common signaling receptor for all GDNF family ligands is the RET (REarrangement during transformation) tyrosine kinase receptor (Durbec et al., 1996; Trupp et al., 1997). All GDNF family ligands first bind to their corresponding GDNF family receptor-α (GFRα) proteins forming a high affinity complex. GDNF, NRTN, ARTN and PSPN specifically bind to GFRα1, GFRα2, GFRα3 and GFRα4, respectively (Airaksinen and Saarma, 2002). The GFRα1-receptor has been shown to exist as bound to the plasma membrane by a glycosylphosphatidylinositol anchor, but it has also been shown to be biologically active in a soluble form (Paratcha et al., 2001). Homodimeric GDNF-family ligand/GFRα-receptor complexes induce dimerization of two RET molecules, leading to autophosphorylation of tyrosine residues in the intracellular domains of RET proteins.

2.5.2 The role of BDNF and GDNF in the models of ischemic brain injury

Numerous studies have reported that exogenous BDNF treatment after acute ischemic insult reduces infarct volume and significantly restores behavior function (Schabitz et al., 1997; Yamashita et al., 1997; Schabitz et al., 2000). Following subcortical ischemic stroke in adult rats, increases in oligodendrocyte differentiation and myelin formation are observed at 7 and 28 days after single-dose BDNF injected at 24h after injury (Ramos-Cejudo et al., 2015). In photothrombotic stroke model, daily intravenous injections of BDNF for five days improves sensorimotor outcomes as assessed by rotarod, balance beam. Implantation of BDNF-transfected fibroblasts into the somatosensory cortex after stroke induced the upregulation of Trkβ receptors in cortical neurons in the penumbra, which increased neuronal survival in the cortex (Ferrer et al., 2001). GDNF has also been shown to have neuroprotective effects following ischemic brain injury when introduced to the brain by viral vectors or GDNF-expressing cells (Duarte et al., 2012). Similar to VEGF, there have been no clinical trials using exogenous BDNF or GDNF astherapeutic agents. The lack of clinical trials may be secondary to difficulty of extracting or producing appropriate amounts of these neurotrophic factors for use in humans (Nagahara and Tuszynski, 2011), as well as questioning of growth factor proteins-induced tumore formation or side effects (Kawai et al., 2000; d'Anglemont de Tassigny et al., 2015).

2.5.3 The role of BDNF for endogenous NPCs in the stroke cortex

Interestingly, BDNF treatment has also been shown to increase neurogenesis in dentate gyrus as well as migration of SVZ progenitor cells to striatum of the injured hemisphere. Previous studies have demonstrated p75NTR expression defines a population of cells in the SVZ that persists into adulthood and is able to respond to stimulation by neurotrophins (Young et al., 2007). It has also been suggested that BDNF induces neurogenesis via p75NTR activation, but also affects dendritic development of SVZ-derived neurons via its high affinity receptor TrkB (Bath and Lee, 2010). While there is evidence in ischemic models for BDNF upregulation within the injury penumbra, the BDNF-mediated effects in the stroke brain are controversial (Galvao et al., 2008). These discrepancies, along with data showing that BDNF modulates other neurogenesis-related

factors such as VEGF, indicate that the effects of BDNF on neurogenesis after stroke may be indirect (Talwar and Srivastava, 2014). Indeed, a recent study has shown that BDNF promotes differentiation and maturation of adult-born neurons indirectly by enhancing the release of GABA from interneurons (Waterhouse et al., 2012). However, several reports support the idea of a direct effect of BDNF on SVZ migration in stroke animals. Systemically applied BDNF has been shown to enhance recruitment of NPCs into ipsilateral striatum after stroke (Schabitz et al., 2007). In addition, Sofia et al. and others have demonstrated that neuroblasts in the damaged striatum express p75NTR and migrate along the astroglial scaffold in response to vasculature-expressed BDNF (Grade et al., 2013). It is suggested that BDNF plays a pivotal role in the switch from the stationary to the migratory phase of neuroblasts by promoting the initiation of migration of cells navigating into the damaged striatum. Nevertheless, TrkB-expressing astrocytes in the ischemic area envelop the vessels and trap BDNF to modulate its availability for neuroblast migration, which may be one reason why injury-induced migration of neuroblasts to the striatum is much slower than constitutive migration in the RMS. Although evidence supports that increasing BDNF has the potential to facilitate the migration of SVZ cells in the stroke brain, it is unclear if this improvement of cell migration is mediated through an action in the SVZ or the target lesioned sites.

2.6 The MANF family of neurotrophic factors

2.6.1 Structure and expression of MANF

MANF and CDNF proteins are produced from homologous genes found in the genomes of vertebrates, such as humans, rodents, and zebrafish (*Danio rerio*). The genomes of invertebrates, fruit fly and nematode, have a single orthologue that is slightly more homologous to human MANF than CDNF genes (Petrova et al., 2003; Lindholm et al., 2007). Analysis of their primary amino-acid sequence predicted the presence of an amino-terminal (N-terminal) signal peptide, four possible intramolecular cysteine bridges and a C terminal ER retention signal that directs them to the ER, which when cleaved can result in a mature protein being secreted (Mizobuchi et al., 2007). MANF was shown to be an intracellular protein co-localizing with markers of the ER and can be secreted in

response to ER stress (Apostolou et al., 2008; Henderson et al., 2013). Crystallographic studies of the structure of human MANF and CDNF revealed that they have two domains (Parkash et al., 2009) (Fig., 5). The folding of MANF and CDNF into two distinct domains linked with a flexible loop, and the conformation of the C-terminal domain was verified with NMR studies of full-length proteins (Hellman et al., 2011; Latge et al., 2015). The N-terminal domain of MANF and CDNF has structural homology to saposin-like proteins, a family of proteins with diverse functions but a shared ability to interact with lipid or membranes (Bruhn, 2005; Parkash et al., 2009). The C-terminal domain of MANF was shown to be similar to the SAP-like domain proteins (SAF-A/B, Acinus, and PIAS) including Ku 70, a protein required for the non-homologous end joining for DNA repair, but also shown to have antiapoptotic properties (Hellman et al., 2011). Moreover, the last four amino acids of MANF are similar and recognized by KDEL-receptors that facilitate the ER retention of proteins from Golgi to ER (Raykhel et al., 2007). Accordingly, the RTDL motif of MANF has been shown to be important for retaining MANF in the ER, as its deletion or mutation increases the secretion of MANF and causes mislocalization from the ER. Deletion of the corresponding KDEL motif of CDNF similarly increases the secretion of overexpressed CDNF (Glembotski et al., 2012; Henderson et al., 2013; Henderson et al., 2014).

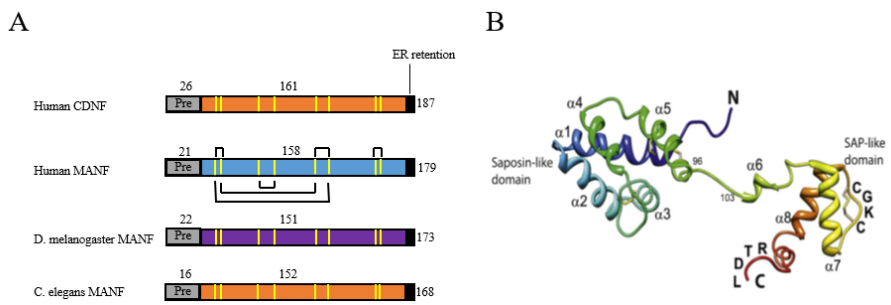

Figure 5. (A) Schematic images showing the position of the signal sequence ("Pre"), conserved cysteine residues (yellow vertical bars), disulfide bridges (black connecting) and C-terminal KDEL receptor-binding motif (black bars) in the primary structure of human CDNF and MANF orthologues from human, *Drosophila melanogaster* and *Caenorhabditis elegans*. (B) NMR solution structure of human MANF consisting of an N-terminal saposin-like domain (residues 1-95) and a C-terminal SAP-like domain (residues 96-158). The hinge region (residues 96-103), CXXC motif and ER retention signal are indicated. N, amino-terminus; C, carboxy-terminus. (A), modified from (Lindholm and Saarma, 2010) (B), adapted

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2.6.2 Regulation of ER stress by MANF

A number of cellular insults including pharmacological perturbation, reduction in ER calcium stores, viral infections, altered protein glycosylation, and increased or unbalanced protein expression can disrupt protein folding and cause accumulation and aggregation of unfolded proteins in the ER lumen. This causes ER stress and if prolonged, leads to ER stress -induced apoptosis. MANF has been shown to be upregulated in cultured cells in response to ER stress-inducing neurotoxins (Holtz and O'Malley, 2003) and other inducers of the UPR, like tunicamycin, an N-glycosylation inhibitor, and thapsigargin, a sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor (Lee et al., 2003; Apostolou et al., 2008; Liu et al., 2016). Also, stress insults or pathological conditions involved in acute or chronic ER stress have been shown to upregulate *Manf* expression *in vivo*. The former includes a tunicamycin-induced UPR in the developing mouse brain (Wang et al., 2015), a mouse model of experimental acute myocardial infarction (Harpster et al., 2006; Tadimalla et al., 2008), and a transient focal cerebral ischemia model (Airavaara et al., 2009). The models of chronic ER stress exhibiting *Manf* upregulation include a pancreatic beta-cell-derived cell line expressing a misfolding-prone version of the proinsulin gene (Mizobuchi et al., 2007) and mouse models of genetic skeletal diseases based on the ER retention of mutant forms of matrilin-3 (MATN3) (Hartley et al., 2013). The presence of an XBP1-binding ER stress response element in Manf´s promoter, and dependence of Manf induction on XBP1 or ATF6, support the observation that Manf upregulation is a genuine part of the UPR. While tunicamycin treatment upregulates the UPR, it does not cause MANF secretion, showing that MANF secretion is not a general response to ER stress (Oslowski and Urano, 2011). The secretion of MANF in response to calcium depletion in the ER caused by thapsigargin is robust. Moreover, MANF's secretion has been shown to be related to two different mechanisms of MANF´s retention in the ER: one determined by the interaction of MANF with GRP78 and the other dependent on the relatively weak C-terminal ER retention signal (Henderson et al., 2013).

2.6.3 Characterization of MANF in genetic model systems

MANF *in vivo* biology was studied at first in *Drosophila melanogaster*. An invertebrate NTF in Drosophila, DmMANF, was identified as a Drosophila ortholog of human MANF. DmMANF was expressed in glial cells and was required for the development of the Drosophila nervous system (Palgi et al., 2009). Genetic ablation of DmMANF produced lethality at the larval stage. Maternal and zygotic DmMANF null mutants showed a complete loss of dopaminergic neurites and a drastic reduction of dopamine levels. These events were followed by degeneration of axonal bundles in the embryonic CNS with subsequent cell death. Genetic rescue experiments showed the restricted expression of human MANF (not expressed in muscles, body fat or gastric caeca) can compensate for the loss of DmMANF. Human CDNF could compensate for the loss of DmMANF, but much less potently than MANF. These developmental studies with DmMANF were complemented with gene expression analysis of two differing DmMANF genetic deletion mutants using DNA microarrays (Palgi et al., 2012). DmMANF deletion led to expression changes in about 40% of known ER / UPR genes supporting a role of MANF in this cellular stress response pathway. In line with this, increased phosphorylation of eIF2α, indicating activated UPR in the $DmManf^{mz\Delta 96}$ embryos, was demonstrated by Western blotting (Palgi et al., 2012). Moreover, gene expression profiling of DmManf mutants revealed altered expression of several genes involved in dopamine uptake, synthesis, and transport, as well as mitochondrial and nuclear genes involved in mitochondrial respiration known to be associated with PD. Expression of genes involved in ATP-binding nucleic acid metabolism, transmembrane transport, exocytosis and endosomal recycling as well as DNA replication were downregulated. Some upregulated genes were clusters of immune and defense response genes and genes associated with lipid metabolism, oxidoreductases and cell death. Interestingly, when DmMANF was overexpressed in a different *Drosophila* line, an increase in the expression of genes involved in oxidation reduction is observed, hinting at the possibility that DmMANF may be involved in protecting dopaminergic neurons from oxidative stress (Palgi et al., 2012).

In zebrafish MANF is similarly widely expressed. Although knockdown of MANF with splicing-blocking antisense oligonucleotides did not result in an apparent phenotype, it decreased brain dopamine levels by about 50% and the number of tyrosine hydroxylase (TH)- and dopamine transporter-expressing cells in certain brain areas (Chen et al., 2012). While studies on the role of MANF in mammalian nervous system development have not been reported yet, the analysis of *Manf* $^{-/-}$ mice has provided a definitive link of MANF to the UPR. With MANF deficiency, adult mice developed type I diabetics due to progressive decreased insulin production and β-cell death. The phenotype of full-knockout animals was similar to the conditional pancreas-specific deletion of MANF (Lindahl et al., 2014). The insulin deficiency results from increased death and decreased proliferation of pancreatic β-cells. This correlates with upregulation of UPR genes (CHOP, GRP78, ATF4 and ATF6), enhanced Xbp1 splicing and increased eIF2α phosphorylation (Lindahl et al., 2014). Notably, upregulation of some UPR markers precedes β-cell loss, indicating that chronic UPR is a cause, rather than a consequence, of β-cell death.

In the mouse model of spinocerebellar ataxia 17 (SCA17), the accumulation of a mutant TATA box binding protein (TBP) leads to cerebellar Purkinje cell degeneration accompanied by decreased expression of MANF in the cerebellum. Overexpression of MANF ameliorates mutant TBP-mediated Purkinje cell degeneration possibly via protein kinase C (PKC)-dependent signaling (Yang et al., 2014a).

Endogenous MANF has recently been reported to play neuroprotective and regenerative roles in the fly and mouse models of retinal injury (Neves et al., 2016). Light-induced damage to mouse retina resulted in the accumulation of MANF-expressing cells also displaying characteristics of innate immune cells, such as microglia and leukocytes of myeloid origin. Upon treatment with recombinant MANF or transplantation of MANF-expressing fibroblasts, the CD11b-positive cells (a marker of microglia) recruited by the damage started to express markers of alternative immune activation (Ym1 and Arginase-1). Remarkably, MANF also had this effect on isolated bone marrow-derived

macrophages. Moreover, light-induced damage to retina was more severe in mice carrying a single functional MANF allele. The protective effect of recombinant MANF on photoreceptors was also seen in two genetic models of retinal degeneration. The MANF-modulated innate immune cells promoting survival of photoreceptors was counteracted by diphtheria-toxin-mediated depletion of CD11b-positive cells or genetic ablation of Cx3Cr1, a chemokine receptor expressed by monocytes/macrophages and resident microglia.

2.6.4 Therapeutic effects of MANF in various lesion models

Studies on the therapeutic efficacy of MANF in animal models of acute or progressive diseases have mostly concentrated on Parkinson's disease (PD), ischemic injury, and degenerative diseases.

There are limited data available concerning MANF's therapeutic properties in animal models of PD. Pretreatment with intrastriatal injection of recombinant human MANF (rhMANF) was shown to rescue TH-positive neurons in the substantial nigra pars compacta (SNpc) from 6-OHDA-induced cell death (Voutilainen et al., 2009). Moreover, injection of rhMANF several weeks after 6-OHDA administration reversed the toxin-induced behavioral changes seen using amphetamine-induced rotations. However, there was no statistically significant reduction of loss of dopamine neurons and degeneration of striatal dopaminergic fibers. Although adeno-associated virus (AAV)-mediated intrastriatal delivery of MANF has recently been reported to protect the SNpc dopamine neurons and to increase the density of striatal TH-positive fibers (Hao et al., 2017), these data were contradicted by observations that intrastriatal lentiviral delivery of rhMANF had no beneficial histological or behavior effects (Cordero-Llana et al., 2015). Similarly, chronic infusion of rhMANF had no effect on 6-OHDA-induced motor deficits or on degeneration of SNpc TH-positive neurons and their striatal afferents. Despite some mechanistic insights into the function of MANF, its mode of therapeutic action in PD models is still obscure.

MANF has also been shown to protect both cardiac myocytes and neurons from death caused by a period of ischemia followed by reperfusion (table 2). For cardiac myocytes, it has been shown that both overexpressed and extracellularly applied mouse MANF protect cultured cardiac myocytes from simulated ischemia. In a follow-up study, intravenous infusion of MANF *in vivo* also protected mouse cardiac muscle from transient coronary artery occlusion (Glembotski et al., 2012). Airavaara and colleagues studied the possible protective effect of rhMANF using a cortical stroke model of distal middle cerebral artery occlusion (dMCAO) (Airavaara et al., 2009). Intracortical injection of rhMANF just before dMCAO limited the size of the infarction in a dose-dependent manner. MANF protected brain tissue by decreasing the number of apoptotic cells, but had no effect on cerebral blood flow (Airavaara et al., 2009). In addition, the administration of MANF exerted a local CNS therapeutic effect, rather than changes in body temperature, blood pressure, or blood gases values. In a subsequent study, AAV-mediated overexpression of MANF had neuroprotective effects on cortical neurons against ischemic injury. Compared to the control group with AAV-GFP, AAV-MANF treated rats had significantly smaller infarction volumes, indicating that overexpressed MANF can also counteract ischemic-induced cell injury (Airavaara et al., 2010). Similarly, intracerebroventricular (i.c.v.) administration of MANF two hours after the MCAO also reduced infarct size and decreased apoptosis, assessed by the number of positive cells after terminal deoxynucleotidyl transferase dUPT nick end labeling (TUNEL) and levels of cleaved caspase-3 (Yang et al., 2014b). Much like the models of myocardial ischemia, the UPR is also known to influence preservation of cell viability in models of cerebral ischemia-reperfusion, but the mechanism of MANF´s protection is still unknown.

Table 2. Therapeutic application of MANF in animal models of ischemic injury

Year	Model	Treatment	Outcome	Reference
2009	Transient distal MCAo	Intracortical injection of rhMANF 20 min before ischemia	Smaller infarct size (+) Enhanced behavior recovery (+) reduced apoptosis in the cortex	(Airavaara et al., 2009)
2010	Transient distal MCAo	Intracortical AAV7-hMANF injection 1 week before ischemia	Smaller infarct size (+) Enhanced behavior recovery (+)	(Airavaara et al., 2010; Glembotski et al., 2012)
2012	Myocardial ischemia/reperfusion	Intravenous rhMANF 24h before injury	Smaller infarct size (+)	(Airavaara et al., 2009; Glembotski et al., 2012)
2014	Transient proximal MCAo	Intracerebroventricular rhMANF, 2h after ischemia/reperfusion	Smaller infarct size (+) Enhanced behavior recovery (+) Reduced apoptosis in the cortex	(Yang et al., 2014b; Matlik et al., 2015)
2015	Transient distal MCAo	Intracortical injection of MANF-ΔRTDL 20 min before ischemia	Smaller infarct size (+)	(Airavaara et al., 2010; Matlik et al., 2015)

A significant loss of pancreatic beta cells in *Manf* $^{-/-}$ mice raised the question of whether therapeutic application of MANF could rescue beta cells. Indeed, AAV-mediated overexpression of hMANF protected pancreatic beta cells from death induced by streptozotocin (a DNA-damaging alkylating agent taken up by pancreatic beta cells). Even though the effects of AAV-MANF treatment on blood glucose and insulin levels were not statistically significant, it did cause an increase in the number of proliferating beta cells, suggesting that MANF has a regenerative effect and therefore might be a potential drug candidate for type I diabetes (Lindahl et al., 2014).

The neuroprotective role of endogenous MANF in the fly and mouse models of retinal injury were discussed above (see section 2.6.2). In addition, MANF delivery increased integration of transplanted photoreceptors into degenerating retinas and restored visual function in the mammalian retina (Neves et al., 2016). Although this study supports the observation that MANF, by modulating the inflammatory microenvironment, improves the integration of photoreceptors in degenerating retinas, it does not exclude the possibility that MANF may act directly on refractory photoreceptors to improve their integration capacities. In summary, currently available studies indicate that treatment based on MANF, either as a therapeutic agent or a factor for improving the efficiency of cell-based therapies, could have a protective and regenerative potential for a number of different diseases.

3 AIMS OF THE STUDY

The overall aim of this work was to investigate the effects of neurotrophic factors on the properties of NSCs/NPCs in homeostatic or pathological conditions, and to use this knowledge for developing more effective stem-cell therapies in injured brains.

Specific aims were:

- To determine the role of MANF in neuronal differentiation and migration in the developing mouse cerebral cortex.
- To determine the mechanisms involved with MANF regulation of neurite outgrowth when NSCs start to differentiate.
- To investigate and compare the effects of GDNF and MANF treatment on adult NSCs/NPCs after focal cortical stroke.
- To investigate whether BDNF has trophic activity in the SVZ to enhance the migration of SVZ cells toward the lesioned area.

4 MATERIALS AND METHODS

Molecular biology experiments	Original articles	My contribution to the experiments
MANF full-ko lines and genotyping	I/II	
Nesting-Cre mouse lines and genotyping	Lindahl. et al. 2014	
Construction of plasmid DNA vectors	II	+
Quantitative reverse transcription polymerase chain reaction (qPCR)	I	+
Cell culture experiments		
Production of lentiviral vectors	II	
Production of AAV vectors	III	
Culturing primary neurons	II	+
Neural stem cell culture	I/II	+
Oxygen-glucose deprivation test	II	+
Subventricular zone explant culture and migration assay	II	+
Flow cytometry	II	+
Immunological methods *in vitro*		
Immunocytochemistry	I/II	+
Enzyme-linked immunosorbent assay	II	+
Western blot analysis	I/II	+
HPG labeling	I	
PI labeling	II	
Immunological methods *in vivo*		
Immunohistochemical staining	I/II/III	+
Immunofluorescent staining	I/II	+
Western blot analysis	I	+
Animal models		
Stereotaxic intracerebral injections	II/III	+
Rat MCAo model of ischemic injury	II/III	+
BrdU labelling	I/II	+
Minipump implantations	II	+
Mouse MCAo model of ischemic injury	unpublished data	+
Behavior tests (a modified Bederson´s neurological test, elevated body swing test, cylinder test and Rotarod test)	III/unpublished data	+
TTC staining	III/unpublished data	+

Imaging		
Fluorescent microscopy	I/II	+
Quantitative image analysis	I/II/III	+

4.1 Published methods

All methods used in my studies are listed in the above table. The more detailed information on materials and methods can be found in the original articles (I, II and III).

4.2 Unpublished methods

4.2.1 The mouse model of permanent middle cerebral artery occlusion (pMCAo)

Adult *Manf* $^{flox/flox(fl/fl)}$ (WT) and *Nesin* $^{Cre/+}$:: *Manf* $^{flox/flox(fl/fl)}$ (MANF-cKO) (8-10 weeks) (Lindahl et al., 2014) were anesthetized with chloral hydrate (0.4g/kg, intraperitoneally) (Sarabi et al., 2003). Chloral hydrate has been a popular hypnotic anesthetic agent used in laboratory rodents. Its advantages include: 1) rapid onset of action; 2) short duration of anesthesia; and 3) stable anesthetic plane. Its disadvantages include a reasonably well-documented association with adynamic ileus (loss of GI motility with consequent fluid sequestration and constipation) in laboratory rats. In the NIH Animal Program's "Anesthesia Guidelines for Rodents", chloral hydrate is specifically listed as an acceptable anesthetic for laboratory rat surgery, provided that the concentration of drug is kept at 4% or lower, that the users provide an acceptable scientific justification for its use in preference to other rodent anesthetics, and that the animal(s) be kept under very watchful observation for any signs of adynamic ileus such as bloating or constipation. Focal cerebral ischemia was produced by permanently occluding the middle cerebral artery (MCAo). Under a microscope, the right temporo-parietal region of the head was shaved, and an incision was made between the right orbit and the right ear in the shape of a “C.” An incision was made superiorly on the upper margin of the temporal muscle. The MCA was observed through the semi-translucent skull. A small burr hole was drilled into the outer surface of the skull just over the MCA. The inner layer of skull and the dura were removed by fine forceps. The MCA was then ligated with 10-0 monofilament nylon

suture and transected superior to the ligation point. The small flap of facial skin was closed with 6-0 nylon suture.

4.2.2 Assessment of cerebral infarction volume

Two days after pMCAo, the infarction area was measured by 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) staining as described previously. Mice were decapitated and the brains were removed and sliced into 1.0-mm-thick sections using an acrylic mouse brain block. The brain slices were incubated in a 2% TTC solution for 15 min at room temperature and then transferred into a 4% paraformaldehyde solution for fixation. The area of infarction in each slice was measured with a digital scanner and Image-Pro Analyzer 7.0. The volume of infarction in each animal was obtained from the sum of infarction areas in all brain slices examined.

4.2.3 Behavioral measurements

The stroke-induced neurological deficits or recovery-promoting effects of treatments were assessed with sensorimotor tests on days 2, 14, and 24 post-stroke: a modified Bederson´s neurological test, elevated body swing test, cylinder test and Rotarod test. In these tests, rats display ischemic injury-induced neurological deficits that resolve spontaneously over time. The elevated body swing test was carried out as described previously (Borlongan et al., 1998). In a short, rats were examined for lateral movements/turning when their bodies were suspended 20 cm above the testing table by lifting their tails. The most marked body asymmetry seen in stroke animals is 20 contralateral turns/20 trials. In normal rats, the average body asymmetry is 10 contralateral turns/20 trials.

Modified Bederson´s score was used to evaluate neurological deficits as described previously (Airavaara et al., 2009). In this test, rats were examined for the degree of abnormal posture when suspended 20-30 cm above the testing table. The following scale was applied:

0= rats extend both forelimbs straight; no observable deficit

1= rats keep the one forelimb to the breast and the other forelimb is straight

2= rats show decreased resistance to lateral push in addition to behavior score 1

3= rats twist the upper half of their body in addition to behavior in score 2

In the cylinder test, the animal was placed inside a transparent vertical tube with a diameter of 35 cm and its movements were video recorded for 5 minutes. The number of first front paw touches to the inner wall of the tube, after rising on its hind limbs, were counted.

Rotarod test: Animals received 3 days of training (twice daily) before MCAo. Each animal was placed in the respective lane, 13.75 inches above the testing platform. For training, the rod was rotating at 4 r.p.m. initially and then gradually accelerated up to 40 r.p.m. The test was performed in this accelerated mode 2 days as well as after 7 and 16 days after MCAo. The cutoff time for each test was 360 seconds. Each animal was tested 3 times per day. The average endurance times (ETime) on the rotating rod in three trials was used for analysis.

5 RESULTS

5.1 The expression of MANF in the developing and young adult brain (I and II)

Previously MANF mRNA and protein expression were detected in neurons of different areas in the mouse brain. In the present study, we utilized specific MANF antibodies, validated by comparing wild-type and *Manf*$^{-/-}$ cortical sections, to analyze the expression of MANF by immunohistochemistry during the development of the mouse cortex from E13.5 to P35 (I, Fig., 1B; II, Fig., IA). We observed high levels of MANF immunoreactivity in the cerebral plate (CP) of E13.5 and E15.5 mouse brains. At E18, when the laminated structure of the cerebral cortex develops, higher immunoreactivity of MANF was found in the middle layer of CP and preserved in the IV/V layer of the cortex in the postnatal brain. In the cortex of the mature mouse brain, MANF was co-localized with NeuN, but not with GFAP, (II, Fig., 1B-C) suggesting that, as earlier shown by Lindholm et al (2008), MANF protein is mainly expressed in mature neurons. In addition to the cerebral cortex, we further investigated if MANF is expressed in the neurogenic area of the mouse brain. From the embryonic stage to adult age, MANF protein was preserved in cells with the VZ as well as the SVZ, and colocalized with nestin and DCX, which are widely used markers for NSCs and neuroblasts, respectively. Importantly, MANF is expressed in BrdU-positive cells, indicating that MANF is not only expressed in post-mitotic neurons but also in mitotic cells in the developing and early adult brain.

5.2 Loss of MANF does not affect the biological properties of NSCs (I)

Since the MANF-expressed VZ and SVZ areas of the brain are composed of NSC/NPCs, we wanted to further study the possible role of MANF in cells in the neurogenic region by setting up primary NSC cultures from E13.5 mouse brains. NSCs are self-renewing, multipotent cells that generate neurons and glia in the nervous system. Importantly, lack of MANF did not affect the viability of NSCs and *Manf*$^{-/-}$ NSCs continue to survive after at least 15 passages in the presence of mitogenic factors. Next, we compared the self-renewal capacity and proliferation rate between each genotype of NSCs. We found that there is no difference in the ratio of BrdU-labeled cells between each group. Also, *Manf*$^{-/-}$ NSCs have shown a similar rate of single cells formation of new neurospheres

compared to WT (wild-type littermate) cells. Next, we studied whether MANF deletion affects the heterogenous population of NSCs and stained WT and *Manf*$^{-/-}$ NSCs with the neural stem cell marker, nestin, and the NPC marker, DCX. There was no difference in the numbers of nestin-positive or DCX-positive NSC/NPCs between the two genotypes. In line with immunostaining results, Western blot analysis showed no difference between the genotypes in the amount of nestin, GFAP, or DCX levels measured from NSCs. Taken together, the above data demonstrated that MANF-deficiency did not affect biological properties of cultivated NSCs, such as heterogeneous populations, self-renewal capacity, viability, and proliferation.

5.3 Loss of MANF interferes with neurite outgrowth during neuronal differentiation (I)

During NSC/NPC differentiation, cellular morphology changes dramatically and there is a requirement for protein synthesis for development of branch sprouting and production of secretory factors. To investigate whether MANF is involved in neuronal differentiation and neurite growth *in vitro*, NSCs were allowed to differentiate in medium without mitogenic growth factors (EGF, FGF-2) for eight days. At DIV1, the WT and *Manf*$^{-/-}$ cells displayed slightly asymmetrical morphology accompanied by short leading processes. At DIV2, WT cells clearly showed neurite outgrowth and there was increased neurite extensions at DIV4. In contrast, *Manf*$^{-/-}$ cells had no obvious neurite sprouting at DIV2 and had decreased neurite length at DIV4 (I, Fig., 3B, C), suggesting that loss of MANF impaired neurite outgrowth during neuronal differentiation. While a single application of rhMANF (200ng/ml) could restore neurite outgrowth of *Manf*$^{-/-}$ cells at DIV2, it did not subsequently increase the neurite length at DIV4. After differentiation, analysis of total neurite length revealed that *Manf*$^{-/-}$ neurons displayed significantly shorter neurites when compared with WT neurons. As neurite outgrowth is a crucial step for neuronal differentiation, we also found that there were significantly fewer TuJ1- and MAP2-labeled cells with neuronal morphology in the *Manf*$^{-/-}$ group when compared to WT cells at DIV8. The number and morphology of GFAP-positive cells did not differ between the two groups (I, Fig., 3D, G), indicating that MANF deletion did not affect gliogenesis. In line with the immunocytochemical staining, Western blot revealed

decreased protein levels of TuJ1 and MAP2, but not GFAP in the *Manf* $^{-/-}$ group (I, Fig., 3I-J).

Given the observed effect of MANF deletion on neuronal differentiation *in vitro*, we next wanted to study whether the lack of MANF disturbs neurogenesis during cortical development. Immunofluorescent staining of neuronal nuclei (NeuN), a marker for postmitotic neurons, microtubule-associated proteins (MAP2), a mature neuron-specific marker confined to the soma and dendrites, and neurofilament (NF-200), a neuronal cytoskeletal protein supporting axon outgrowth, was carried out. At E19 when cortical lamination is developing, few NeuN-positive neurons and MAP2-positive dendrites were located at the CP in the WT brain. Since *Manf* $^{-/-}$ cortex was much thinner, NeuN-positive neurons were scarce and tiny MAP2-expressing neurites were visible (I, Fig., 8A-C). When CP tissue lysates were analyzed by Western blotting, there were no significant differences in relative levels of NeuN and MAP2 between each genotype. However, the level of neurofilament (NF200) was lower in *Manf* $^{-/-}$ CP (I, Fig., 8D and E). At P7, when cortical neurons have already moved to their destination and develop axonal elongation and dendritic branching, there was no difference in the total number of NeuN-positive neurons between the two genotypes, but increased neuronal density in the layers II-V of *Manf* $^{-/-}$ cortex was observed (I, Fig., 8F-G). In WT neurons MAP2 staining was detected in the cell soma and dendrites. In contrast, less MAP2 expression in *Manf* $^{-/-}$ neurons was observed and was limited to the soma (I, Fig., 8I and J). Furthermore, axons from *Manf* $^{-/-}$ cortex stained less intensely with the neurofilament marker NF200 compared to WT axons (I, Fig., 8K and L). In line with immunostaining data, the level of NF in Western blot analysis was reduced by approximately 35% in *Manf* $^{-/-}$ cortex compared to WT (I, Fig., 8M and N). Thus, consistent with *in vitro* data, loss of MANF during cortical development also disturbed dendritic/axonal outgrowth and predominantly decreased NF production.

5.4 Loss of MANF results in activation of UPR and decreased *de novo* protein synthesis (I)

Previous studies have indicated that MANF is upregulated under ER stress conditions *in vitro* (Apostolou et al., 2008; Glembotski et al., 2012) and lack of MANF leads to chronic UPR activation in pancreatic islets *in vivo* (Lindahl et al., 2014). Therefore, we hypothesized that the deficits of neuronal differentiation from *Manf* $^{-/-}$ NSCs may result from aberrant ER activity. First, we studied the level of ER stress and UPR activation in WT and *Manf*$^{-/-}$ cells during neuronal differentiation. At DIV1, the levels of spliced *Xbp1* mRNA expression were higher in the *Manf* $^{-/-}$ group, followed by increased Grp78 at DIV4 (I, Fig., 9A, and C). At DIV 8, significant elevations of mRNA levels for *Grp78, Atf4,* and spliced *Xbp1* were found in *Manf* $^{-/-}$ differentiated cells (I, Fig., 9D). Also, quantification of phosphorylated (p)eIF2α band intensities compared to total levels of (t)eIF2α by Western blot analysis revealed a higher level of phosphorylated eIF2α in *Manf* $^{-/-}$ cells at DIV8 (I, Fig., 9E and F), suggesting loss of MANF indeed results in an activated UPR during neuornal differentiation.

Since ER function is crucial for protein synthesis and processing and UPR attenuates protein synthesis, we wanted to study whether MANF deficiecy also disrupts protein synthesis when NSCs differentiate. We utilized a homopropargylglycine (HPG)–based assay for the detection of nascent protein synthesis. At DIV1, most WT and *Manf* $^{-/-}$ NSCs showed strong HPG-derived signals. However, at DIV2, there was less HPG-derived signal in *Manf* $^{-/-}$ cells (I, Fig., 9J and K). Importantly, the fluorescent signals in WT cells were spread to growing neurites. In contrast, the signals in *Manf*$^{-/-}$ cells were limited to the soma, suggesting that most *Manf* $^{-/-}$ cells have not yet developed neurite sprouting. Collectively, these data demonstrated that deficiency of MANF disturbs ER homeostasis, including activated UPR, and further decreases protein synthesis.

5.5 Delayed neuronal migration in MANF-deficient embryos (I and II)

Neural migration, the movement of neuronal precursors from their sites of origin in the proliferative layers to their ultimate locations in the mature brain, is a critical step in the development of the vertebrate nervous system. Neuronal migration is most likely

regulated by different types of extracellular signaling molecules that cells encounter as they migrate (Mason et al., 2001). Moreover, cellular movement mechanisms and neurite outgrowth play critical roles in initiating and modulating the movement of neuronal precursors (Lee et al., 2012). Given that MANF is implicated in axonal outgrowth, we wanted to study if MANF-deficiency affects SVZ cell migration. We isolated the SVZ from WT and *Manf*$^{-/-}$ embryos (E19) and cultivated these in Matrigel cultures to investigate cell migration out of SVZ explants. Cell migration was examined from WT and *Manf* $^{-/-}$ SVZ explants *in vitro*. On DIV2, there was little cell migration and no difference between each group. On DIV7, *Manf*$^{-/-}$ SVZ cells had shown shorter migration distances compared with WT cells (II, Fig., 2J and K).

Next, we utilized BrdU birth dating to examine neuronal migration in the developing cortex. To study the migration of neurons, we injected pregnant *Manf* $^{+/-}$ mice with BrdU at E15.5 and analyzed the embryos at E19. The numbers of Brdu-labeled cells in each layer was counted and documented as percentages of total labeled cell numbers. The relative number of BrdU-positive cells in the layer V/VI of *Manf* $^{-/-}$ embryos was significantly increased, and was decreased in layer II/III compared to WT littermates (I, Fig., 7). Taken together, we demonstrated that MANF is essential for the migration of neurons in the embryonic cortex.

5.6 Loss of MANF caused larger infarct volume and increased NSC vulnerability to OGD/reoxygenation-induced stress (unpublished data, II)

While MANF has been shown to be protective of cerebral neurons from ischemic injury in the model of transient MCAo in rat, it is not clear if MANF's effect is mediated through a direct action on neurons or microenvironment modulation. In this work (unpublished data), we established the permanent MCAo model of cortical stroke and analyzed infarct volume in adult *Manf* $^{flox/flox(fl/fl)}$ (WT) and *Nesin*$^{Cre/+}$:*Manf* $^{flox/flox(fl/fl)}$ (MANF-cKO) (8-10 weeks). By using TTC staining on day 2 post-stroke, MANF-cKO mice displayed a larger infarction volume than their WT littermates (Fig., 6), suggesting that endogenous MANF has the potential to protect cortical neurons from ischemic injury.

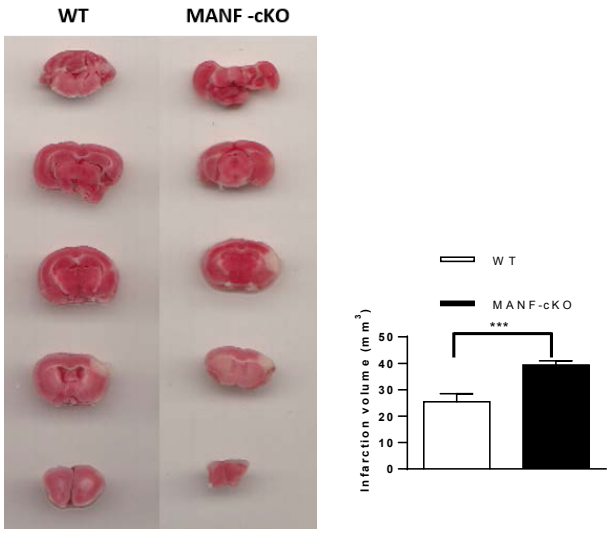


Figure 6. TTC staining on day 2 after dMCAO. MANF-cKO mice displayed a larger infarction volume than the WT littermates. Values shown are the mean± SD. ***p< 0.001 between WT and MANF-cKO mice (n=7-9).

To further study whether MANF also has a potential to protect NSCs from stress insults, we set up the hypoxic model with 40-min oxygen-glucose-deprivation (OGD) followed by 24-hours reoxygenation. Propidium iodide (PI) was detected by means of flow cytometry in *Manf*$^{+/+}$ - and *Manf*$^{c/-}$-NSCs exposed to OGD/reoxygenation. PI is a fluorogenic compound that binds stoichiometrically to nucleic acids so that fluorescence emission is proportional to DNA content of a cell (Wallen et al., 1982). When apoptotic cells are stained with PI and analyzed with a flow cytometer, they display a broad hypodiploid peak, which can be easily discriminated from another peak of cells with normal (diploid) DNA content in red fluorescence channels (Ormerod et al., 1992). In normoxic conditions, the percentage of PI-positive cells were not significantly different between *Manf*$^{+/+}$ and *Manf*$^{c/-}$ NSCs (Fig., 7A& B; 24.85%±2.85 (n=6) vs 25.16%±1.32 (n=6), respectively). In *Manf*$^{c/-}$ NSCs, 40 minutes of OGD followed by 24 h of reoxygenation caused a significantly higher increase in the number of PI-positive cells, compared to *Manf* $^{+/+}$ NSCs (Fig., 7C& D; II, Fig. 2J). Next, we assessed whether recombinant human MANF (rhMANF) can rescue OGD-induced apoptotic cells. Compared to *Manf* $^{-/-}$ NSCs, pre-treatment of MANF significantly decreased the

percentage of PI-positive cells (Fig., 7E& F; II, Fig. 2J), implying loss of MANF increases NSC vulnerability to OGD stress. However, in WT cells, the administration of MANF did not improve cell viability. Therefore, the above data suggests that loss of MANF increases OGD-induced cell apoptosis and administration of rhMANF could, at least partially, restore the function of MANF in *Manf* $^{-/-}$ NSCs.

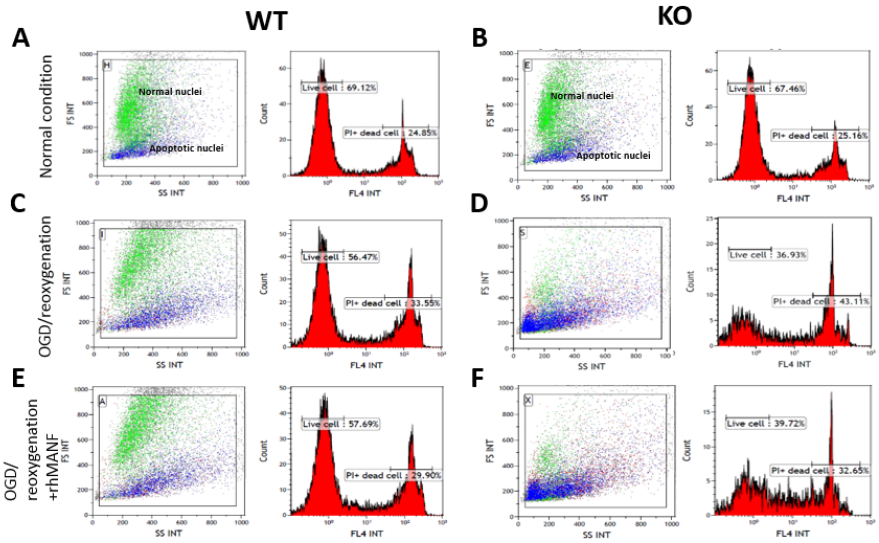


Figure 7. Analysis of NSCs apoptosis by propidium iodide (PI) staining and flow cytometry. The left panel shows that dot-plot analysis of WT (A) and *Manf* $^{-/-}$ (B) NSCs/NPCs in the normal condition undergoing apoptosis. Apoptotic cells can be recognized by lower diameter (FS) and increased DNA fluorescence compared to normal cells. The right panel reveals a precise and reproducible estimate of percentage of normal and apoptotic nuclei, which can be obtained by analysis of the DNA histogram. The flow cytometric and DNA histogram plots show the presence of more characteristic hypodiploid nuclei when death of *Manf* $^{-/-}$ cells was produced by OGD treatment (D), compared to WT group (C). The normal/apoptotic nuclei of MANF-treated WT (E) and *Manf* $^{-/-}$ (F) NSCs, induced by OGD/reoxygenation, are shown in flow cytometric plots and DNA histogram of the peaks corresponding to the normal and apoptotic nuclei.

5.7 Administration of MANF induces differentiation of NSCs (II)

Our studies, described above, show that loss of MANF leads to disturbed neuronal differentiation, including shorter neurite outgrowth. Next, we wanted to study if exogenous application of rhMANF affects the biological properties of NSCs. To investigate the effects of extracellular MANF, rhMANF (400 ng/ml) or vehicle (PBS) as

added to the WT and *Manf*$^{-/-}$ NSC cultures from DIV1-4. First, we analyzed the diameter of neurospheres and found no difference in the size of neurospheres between each group. However, many rhMANF-treated WT and *Manf*$^{-/-}$ NSCs showed localized budding and cellular processes attaching to the surface of petri dishes, implying that these changes preceded morphologic changes. Immunofluorescent staining revealed that there was similar immunoreactivity for nestin, a neural stem cell marker, between each group. There was strong expression of TuJ1, a marker of immature neurons, and GFAP, a marker for glial cells, in the rhMANF-treated neurospheres (II, Fig., 2E and F). Western blot analysis also showed increased levels of neuron-specific tubulin and glial-associated intermediate filament in the MANF-treated group. Accordingly, our data demonstrated that exogenous application of MANF did not affect neurosphere formation derived from proliferating NSCs, but induced filopodia changes accompanied by increased neuronal- and glial-associated cytoskeletal proteins which is crucial for the early step of neuronal/glial differentiation.

5.8 MANF promotes migration of SVZ cells (II)

Since endogenous MANF is essential for embryonic SVZ cell migration, we wanted to study if administration of MANF further promotes cell migration from postnatal SVZ explants. First, we added rhMANF protein (200, 400 and 800ng/ml) to the SVZ explant cultures from DIV1 to DIV5. On DIV7, we found that application of rhMANF (200 ng/ml and 400 ng/ml) increased the cell migration distance from SVZ explants, when compared with the vehicle (PBS) group (II, Fig., 4C, D). However, 800 ng/ml rhMANF did not have a significant effect on SVZ explants, implying that exogenous MANF regulated the migration of SVZ cells in an inverted “U” dose-dependent manner.

Next, we wanted to study whether endogenous overexpression of MANF in NPCs has similar effects on cell migration. Therefore, we produced lentiviral vector (LV)-mediated overexpression of MANF that contains RTDL (hMANF) or lacks the RTDL sequence (deltaRTDL). To verify the transduction of viral vectors, we transduced developing cortical neurons with LV-hDCX-GFP, LV-hDCX-hMANF-GFP, or LV-hDCX-MANF

(deltaRTDL)-GFP. Using a sensitive enzyme-linked immunosorbent assay (ELISA), we found that 5 days after transduction by LV-hDCX-hMANF-IRES–GFP, LV-hDCX-MANF (deltaRTDL)-GFP, the levels of hMANF in developing cortical neurons and media were significantly increased compared with naïve cells or cells transduced by LV-hDCX-GFP (Fig., 8A). Interestingly, the developing cortical neurons transduced by LV-hDCX-hMANF-IRES–GFP and by LV-hDCX-MANF (deltaRTDL)-GFP had similar hMANF concentrations in the medium (Fig., 8B), suggesting that deletion of the RTDL sequence does not increase the secretion of hMANF from cells under these conditions. The cultured SVZ explants were transduced by LV-hDCX-GFP, LV-hDCX-hMANF-IRES-GFP, or LV-Dcx-hMANF (deltaRTDL)-IRES-GFP on day 1 (DIV1). On DIV6, overexpression of MANF induced a longer distance of cell migration than the naïve or GFP group (Fig., 8C). Moreover, overexpression of MANF-deltaRTDL showed similar effects on SVZ cell migration, implying that the RTDL motif is not required for MANF-promoted SVZ cell migration.

Next, we investigated which molecular mechanism is involved in the MANF-promoted migration of SVZ. We found overexpression of MANF significantly activated the p-ERK/ERK pathway and induced the phosphorylation of STAT3-SER727 on DIV4 (II, Fig. 6G, I and J-L), but had not increased p-AKT and p-S6K levels (II, Fig. 6H, I). In addition, there was no difference in GRP78 protein levels between groups at any time points, though MANF has been implicated in regulating UPR markers. Together, these results suggest that overexpressed MANF in DCX^{+} cells would induce STAT3 and ERK1/2 activation when cells start to migrate from SVZ explants.

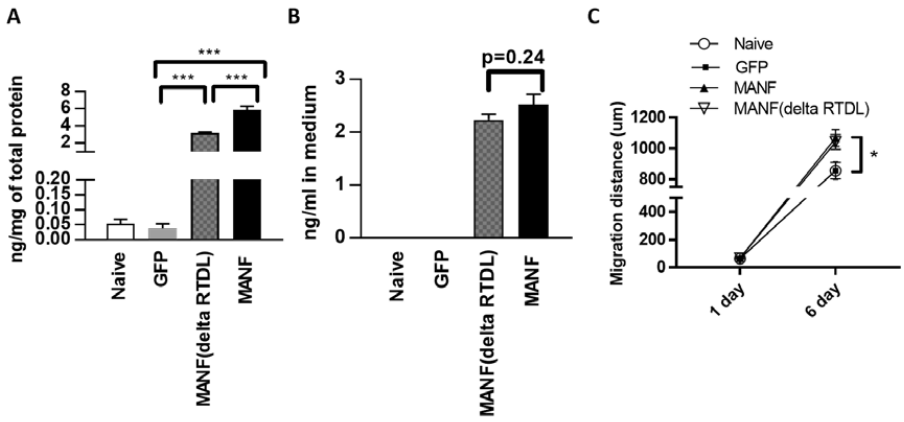


Figure 8. Transduction of SVZ cells with LV-MANF or LV-MANF (delta RTDL) enhances cell migration from SVZ explants derived from P1 mouse brain. MANF protein concentration in cell lysates (A) and culture media (B) from embryonic cortical neurons transduced with indicated LVs (1-way ANOVA, *** $p<0.001$ compared to GFP, TK). C, Quantified migration distance at DIV1 in all groups (n=4-5) and at DIV6 revealed increased cell migration from SVZ explants transduced with LV-MANF and LV-MANF (delta RTDL) as compared to the Naïve, GFP groups (n=11-12, 1-way ANOVA, $p=0.02$; * $p<0.05$). Data are expressed as the Mean ± S.E.M.

5.9 MANF promoted the migration of DCX^+ cells toward the infarct boundary (II)

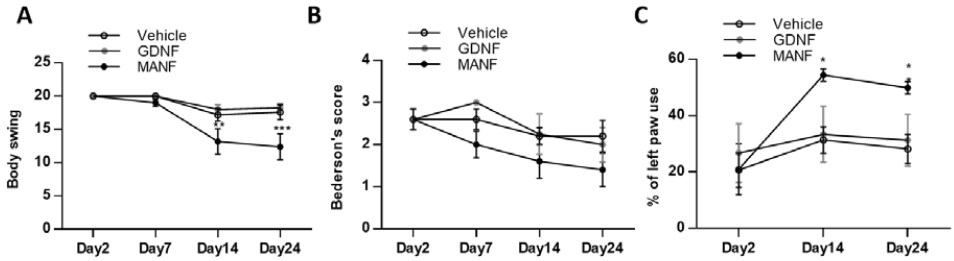
Since our studies showed that MANF treatment induced SVZ cell migration in vitro, we wanted to study if application of MANF protein could also have the potential to promote NPC migration toward the ischemic area after cortical stroke. Stroke rats were stereotaxically injected into right lateral ventricles with GDNF, rhMANF or vehicle (1x PBS) on days 3, 7 and 10 after dMCAo. As expected, GDNF treatment increased density of DCX immunoreactivity (neuroblasts) in the ipsilateral SVZ. In contrast, MANF treatment did not increase the number of DCX^+ cells in the SVZ compared to the vehicle group. However, application of rhMANF significantly increased the numbers of DCX^+ cells in the corpus callosum and infarcted neocortex (II, Fig., 7J-M). Additionally, MANF induced large chains of neuroblasts with long, branched processes in the corpus callosum beneath the infarction zone. Taken together, the above data revealed that the administration of MANF protein into the right lateral ventricle facilitated migration of neuroblasts to the injured cortex.

We next explored whether MANF, compared to GDNF, influences the fate of newly generated cells 14 days after cortical stroke. MANF treatment markedly increased the number of cells labeled with BrdU and DCX in the corpus callosum beneath the infarction zone, compared to vehicle and the GDNF-treated group (II, Fig., 8A, B). There was a trend towards fewer cells double-labeled with BrdU and the astrocyte marker GFAP, in the infarcted area of neocortex after MANF treatment (II, Fig., 8C, D). Meanwhile, the number of BrdU+ cells co-expressing the oligoprogenitor cell marker NG2 was not different among the three groups (II, Fig., 8E, F). Thus, the administration of MANF after cortical stroke may have selectively affected the migrating immature cells of the neuronal lineage.

5.10 Long-term infusion of MANF increased recruitment of DCX^+ cells in infarcted cortex and accelerated behavioral recovery (II, unpublished data)

After determining the effects of MANF and GDNF on DCX^+ cell migration in cortical stroke, we next examined the efficacy of long-term treatment of MANF or GDNF for experimental ischemic stroke. Therefore, using osmotic minipumps, GDNF, MANF, or vehicle were infused into the peri-infarction zone for post-dMCAo days 3-16 and the rats were euthanized one week later (II, Fig., 9A). Lesion size did not differ among three groups. As expected, GDNF, a classical chemoattractant, increased the number of DCX^+ cells in the infarct area of the striatum (Kobayashi et al., 2006). Interestingly, MANF treatment also promoted the recruitment of neuroblasts in the lesioned cortex (II, Fig., 9H and I). Then, we continued to explore whether MANF or GDNF increased the differentiation of progenitor cells into cortical neurons by labeling them with BrdU, and with a marker for mature neurons (NeuN). MANF or GDNF treatment failed to further increase the number of newly-generated cortical neurons (Brdu+/NeuN+ cells) in the peri-infarct area on day 24 (II, Fig., 9J and K). Finally, we analyzed the behavior of rats after administration of MANF and GDNF in the peri-infarct area (unpublished data). All stroke rats were examined on days 2, 7, 14 and 24 after MCAo. Before treatment, all animals demonstrated close to 100% body asymmetry at 2 days after pMCAo. In MANF-treated rats, there was a significant reduction in body asymmetry on day 24 post-stroke

(Fig., 9A). However, in GDNF-treated rats, there was no improvement in body asymmetry at different time points. Modified Bederson's neurological test was carried out on days 2, 7, 14, and 24 after MCAo. In stroke animals, no significant difference was found among each group at different time points, though the MANF treatment had a trend towards reduced Bederson's score on day 24 post-MCAo (Fig., 9B). Cylinder test studies involved the use of contralateral paws (i.e. left) evaluated for 10 minutes on day 2, 14, 24 post-stroke. In cylinder test, the MANF-infused rats showed, compared to the PBS- or GDNF-infused rats, a higher frequency of using their left forepaw to touch the plexiglas cylinder on both days 7 and 14 post-stroke (Fig., 9C). Taken together, these data suggest that chronic infusion with MANF, but not GDNF, could induce behavioral improvement in stroke animals.

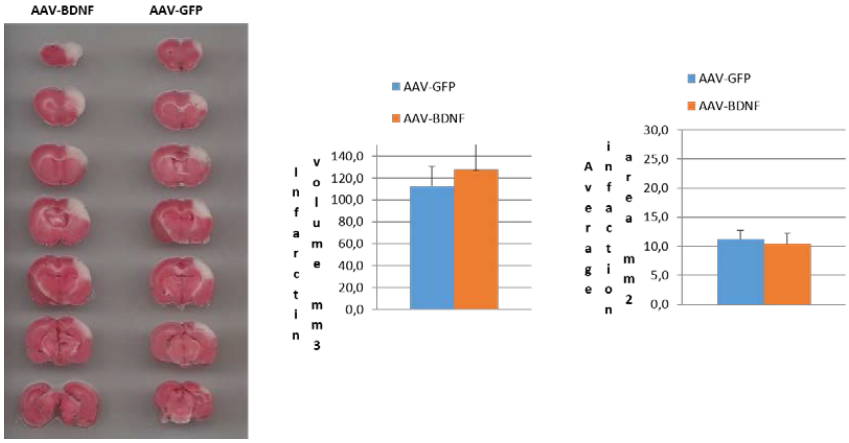


Figures 9. Long-term infusions with MANF after stroke induces behavioral recovery. The severity of their neurological deficit was assessed on days 7, 14, and 24 using the body swing test (B) and Bederson's neurological test (C). According to two-way ANOVA (F (6, 46) = 2,527; P=0.033), the difference in the number of body turning to contralateral side of the lesion were statistically significant on day 24 after stroke. The percent of left forepaw use of PBS, GDNF, and hMANF-infused rats as determined using the cylinder test. According to two-way ANOVA (F (2, 36) = 3,532; P=0.039), the differences in left forepaw use were statistically significant. Chronic infusions of MANF significantly decreases left forepaw use on days 14 and 24 after stroke. Data are expressed as the Mean ± S.E.M.

5.11 Pre-stroke administration of AAV-BDNF to the contralateral SVZ did not alter the size of infarction, but induced NPC migration to the lesioned hemisphere (III)

Abundant experimental data have supported the idea that BDNF has beneficial effects in animal models of stroke. For example, intraventricular administration of BDNF was shown to reduce infarct size after focal cerebral ischemia in rats. Similarly, pretreatment with AAV (serotype 2)-BDNF, given i.c.v., had a neuroprotective function. However,

these data were contradicted after the systemic administration of BDNF, which did not affect infarct size. This discrepancy indicated that the neuroprotective function of BDNF is dependent to the administrative route or location. To better understand the therapeutic effect of BDNF, AAV-BDNF or AAV-GFP was injected locally to the left SVZ at 14 days prior to the right MCAo in rats. We found that AAV-BDNF delivered to the SVZ contralateral to MCAo did not alter the size of infarction (Fig., 10); however, there was enhanced endogenous NPC migration from the SVZ toward the lesioned hemisphere. (III, Fig., 3 and 4). Therefore, these data demonstrated that BDNF's trophic response is limited to the site of injection.



Figures 10. TTC staining on day 2 after dMCAO. AAV-BDNF treatment displayed a similar infarction volume compared to the AAV-GFP. Values shown are the mean± SD. (n=7-9).

6 DISCUSSION

6.1 A critical review of the methods used

6.1.1 Neurosphere culture

The neurosphere culture is a widely used cell model for studying the biological properties of NSCs, such as proliferation and differentiation *in vitro*. Much of the currently available information regarding these features derive from studies utilizing this cell model. In this study, NSCs from embryonic mice were used. Mouse embryos are easily available and the preparation of cortical progenitors from embryonic mice is straightforward. The mouse is also a good model to study human development and disease as it shows considerable similarity to human anatomy and physiology, including the structure and development of the cortex (Ayala et al., 2007; Bystron et al., 2008). The use of mice also allows for modification of the mouse genome, something that is not easily available in humans. In this study, we utilized mice with ablation of the *Manf* full-length transcript for studying the role of MANF in NSC development.

Isolation of embryonic stem/progenitor cells by dissection of the dorsal wall of the lateral ventricles were used followed by enzymatic dissociation and subsequent rounds of centrifugation through various solutions to purify the NSCs. After dissociation, the NSCs/NPCs were grown in suspension culture in the presence of mitogens. Any contaminated cells adhering to the bottom of the culture vessel were removed upon re-plating of the cells in a new culture vessel. Just like with other cell culture systems, the neurosphere culture system has its advantages and disadvantages (Jensen and Parmar, 2006). The proliferation of cells is affected by cell plating density (Tropepe et al., 1999). In addition, the composition of growth media and concentration of intrinsic and extrinsic factors, such as EGF and FGF, affect cell proliferation and differentiation (Irvin et al., 2003). The method of passaging (enzymatic or manual) may also affect the properties of cells, as do the passage number as the cells tend to lose their neurogenic potential, and become more gliogenic upon long-term cultivation. The reason for this remains unknown. We made use of the fact that neurospheres can be frozen, thus allowing us to freeze neurospheres at early passage to be thawed when the existing culture grew older.

The high sensitivity of the culture system to the external environment may lead to difficulties in comparing research results obtained by different laboratories. The neurospheres are also heterogeneous in nature, consisting of cells at various stages of differentiation (Jensen and Parmar, 2006). Thus, one should not consider the neurosphere system as a model for studying pure stem cells but as a model to study various stem/progenitor cells arising within the neurospheres. Nevertheless, the neurosphere system does provide a valuable model to study neural development *in vitro*. Importantly, it has been demonstrated that cortical progenitor cells expand as neurospheres in a similar manner as they do *in vivo* (Shen et al., 2006). The intrinsic properties of neurospheres, as well as the fact that they are grown in defined conditions, provide a model system that allows for the study of forebrain development, adult neurogenesis, and factors affecting these processes in cell cultures.

6.1.2 SVZ explant culture

In this study, we used SVZ explant cultures to assess the role of MANF in regulating the intermediary stage of neural/progenitor cell migration. There are a number of advantages in using explant cultures over *in vivo* and other *in vitro* methods. First, cells in explanted tissues are accessible to experimental manipulations easily (Chen et al., 2005). Second, the fact that explants maintain the complex cellular architecture of developing neural tissue means that cell-cell interactions can be studied (Yin et al., 2016). Third, since we can control the chemical composition of the culture medium, we can use explants to test the effect of specific compounds on tissue development (Mani et al., 2010). Nevertheless, since cells are removed their natural environment, this culturing system relies on the presence of growth factors to maintain undifferentiated status. Withdrawal of these induces rapid differentiation into mature cells. This condition limits the ability to analyze the factors that regulate type A neuroblasts, a transient stage between the stem cell and the neuron. To counteract this limitation, pieces of SVZ-derived tissue can be harvested and cultured as explants in a Matrigel containing laminin and collagen, which maintains the neural stem cells in their neuroblast condition and allows them to migrate (Ward and Rao, 2005; Dixon et al., 2015).

6.1.3 Rodent models of focal cerebral ischemic injury

There are many animal models of focal cerebral ischemic injury available. While rats have been a commonly used species for preclinical neuroregenerative trials for ischemic injury, mice with genetic modifications also provide an excellent approach to help determine potential mechanisms of injury and neuroprotection (Sommer, 2017).

Most cases of human ischemic stroke are caused by occlusion of the MCA or one of its branches (Carrera et al., 2007). This is reflected in animal models of stroke where MCAo is the most widely used technique. Recently, focal ischemia induced by thromboemboli has also been of interest because of its resemblance to human ischemic stroke and its role evaluating thrombolytic therapy (Sicard and Fisher, 2009). However, the infarct size is variable and ischemia caused by multiple small clots do not mimic typical clinical ischemic stroke (Sicard and Fisher, 2009). Therefore, the size and characteristics of blood clots are crucial in this model. Another common way of producing MCAo in rats and mice is by the use of an intra-arterial filament. This method results in relatively low animal-to-animal variation in infarct size. However, this approach causes a bigger injury that compromises the striatum, cortex and even thalamus/hypothalamus. Injury to the hypothalamus is rare in human cases, and it also causes hyperthermia that may interfere with the analysis for neuroprotective efficacy of drugs (Li et al., 1999).

In this study, we have used the craniotomy model of distal MCAo together with the bilateral occlusion of the common carotid arteries to reduce collateral blood flow (namely the three-vessel occlusion model) (Durukan and Tatlisumak, 2009). The craniotomy model requires removal of a piece of skull overlying the MCA so that it affects intracranial pressure and BBB integrity as well as causing a small amount of subarachnoid hemorrhage around the MCA trunk. However, it provides a direct exposure window to ligate the distal part of the MCA, which supplies the neocortex. Simultaneous occlusion of the MCA and the ipsilateral common cerebral artery, combined with transient occlusions of the contralateral common cerebral artery, leads to good reproducibility of infarcts (Tamura et al., 1981). This distal MCA occlusion model produces an infarct core in the frontal and parietal cortex, and an evolving infarction over

3-4 days in the adjacent temporal and cingulate cortex as well as the dorsolateral striatum. The injury is characterized by leukocyte infiltration, cytokine production, caspase activation and apoptosis (Durukan and Tatlisumak, 2009; Sommer, 2017). Additionally, in this MCAo model the occlusion can be either transient or permanent. To maintain the infarct cortical size, we have chosen to use a transient 90-minute occlusion in our rat model. For transgenic mice, we also adapted a standardized protocol for performing permanent distal MCAo, which produces an infarct predominantly restricted to the barrel region of the somatosensory cortex.

6.2 The neuroregenerative effect of BDNF after stroke

Experimental data has supported the idea that BDNF has beneficial effects in an animal model of stroke, reducing the size of brain infarction and improving functional recovery (Schabitz et al., 1997). BDNF has also been shown to enhance the neurogenesis and the migration of NPCs in SVZ in non-lesioned animals; the migration is mainly toward the olfactory bulb (Chiaramello et al., 2007). Limited reports indirectly support the effect of BDNF on SVZ cell migration in stroke animals. For example, systemic administration of BDNF enhanced recruitment of NPCs into ipsilateral striatum after stroke (Schabitz et al., 2007). However, systemically applied BDNF can interact with multiple anatomical sites. It is not clear if the improvement in cell migration is mediated through an action in SVZ or the target of lesioned sites. In this study, we demonstrated local AAV-BDNF delivered to the SVZ contralateral to MCAo did not alter the size of infarction, but directly enhanced endogenous NPC migration from SVZ as well as improved functional recovery in stroke animals. These results may provide a new treatment target in non-lesioned side SVZ for stroke patients through gene or recombinant protein therapy.

6.3 The role of MANF in ER function during neuronal differentiation

In this study, we discovered that when cultured in vitro, MANF-deficient cells have impaired neurite outgrowth during neuronal differentiation. In contrast to other classical neurotrophic factors such as GDNF and BDNF, MANF is an ER luminal protein, which is secreted in response to ER stress in vitro (Hellman et al., 2011). Thus, MANF can be

considered an ER-stress-inducible protein which has a protective effect under brain ischemic, neurodegenerative, and apoptotic conditions (Apostolou et al., 2008; Airavaara et al., 2009; Voutilainen et al., 2009) . During neuronal differentiation, neurites acquire their morphology by considerable branch sprouting, which comes with an increased need for protein production (Godin et al., 2016). Increased protein synthesis and accumulation of unfolded or aggregated proteins triggers the UPR, a homeostatic signaling pathway, to cope with the increased demand for protein folding. However, constitutively increased ER stress and activated UPR disturb homeostatic signaling pathways in the ER which can cause aberrant neuronal differentiation, impair neurite outgrowth, and may even result in cell death (Kawada et al., 2014; Kawada et al., 2016). Accordingly, the level of ER stress and downstream UPR signals are critical for neuronal differentiation (Kurosawa et al., 2007). In our experiments, aberrant UPR signals and decreased protein synthesis were observed during *Manf* $^{-/-}$ NSC differentiation. While the downstream targets of the UPR pathways are not well studied in differentiating neurons, increased eIF2α phosphorylation, known to lead to a global decrease in mRNA translation initiation (Lindahl et al., 2014), and ATF4 up-regulation, which disrupts cAMP–induced responses, play crucial roles in neuronal differentiation (Roffe et al., 2013). Accordingly, we assumed that endogenous MANF acts like a chaperon in the ER to fine-tune downstream UPR signals and preserve ER homeostasis, which is crucial for nascent protein synthesis and processing during neuronal differentiation and neurite growth (Fig., 11).

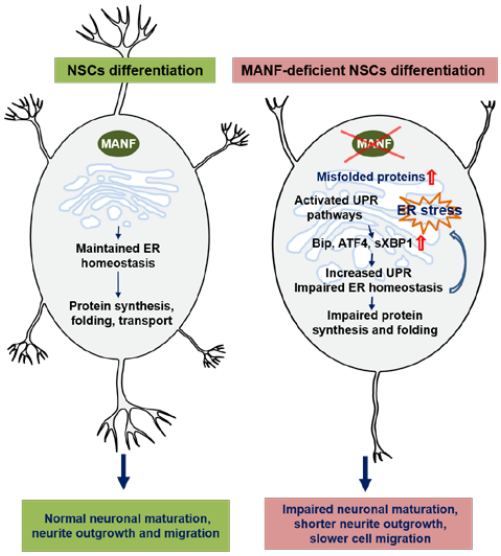


Figure 11. Schematic role of MANF in ER homeostasis during neuronal differentiation, maturation and migration. Adapted and reprinted by permission from Society for Neuroscience.

6.4 The role of endogenous MANF in corticogenesis

Drosophila melanogaster embryos lacking both maternal and zygotic DmMANF show axonal degradation of dopamine neurons (Palgi et al., 2009). However, it is important to note that DmMANF is predominantly expressed in glial cells surrounding dopaminergic neurons, which indicates that the neuronal maturation-promoting factor in the fly orignates from glial cells (Palgi et al., 2009; Palgi et al., 2012; Lindstrom et al., 2016). In contrast to invertebrate species, MANF protein in the mammalian brain is widely localized in NSCs, NPCs, and immature/mature neurons, but not in glial cells, suggesting that MANF is predominantly expressed in the neuronal lineage cells of the mammalian cortex (Lindholm et al., 2008; Wang et al., 2014). Similar to the results seen *in vitro*, deletion of MANF in the developing cortex causes a deficit in neurite outgrowth, especially neurofilament-expressed axonal extension. However, there is no decrease in the generation of cortical neurons in the mutant cortex despite delayed subtype neuron specification and abnormal neuronal density due to a 10% reduction of *Manf*$^{-/-}$ cortical thickness. To elucidate more fully the effects of MANF on cortical development, we also quantified relative levels of GRP78, (p)eIF2α in relation to (t)eIF2α and CHOP by

Western blot analysis in the CP at E19 and in the cortex at P6 (unpublished data). Higher level of GRP78, but not (p)eIF2α related to (t)eIF2α was observed in *Manf*$^{-/-}$ CP at E19. By P7, both of GRP78 and (p)eIF2α were shown to be significantly increased in *Manf* $^{-/-}$ cortex. Similar to *in vitro* data, UPR activation is progressive and constitutive during cortical development. Accordingly, we assumed that *in vivo* there might be another compensating mechanism, as yet unknown, to reverse an aberrant UPR-induced disturbance during *Manf* $^{-/-}$ neuronal maturation. Future studies will be required to address a more mechanistic understanding of corticogenesis as related to ER stress.

6.5 The role of MANF in neuronal migration

In this study, we demonstrated that MANF is required for the migration of neurons to their final destinations in specific layers of the cortex. In contrast to reeler and related mutant mice (Mimura et al., 2008), the development of *Manf* $^{-/-}$ cortex shows an inside-out laminar formation pattern. Additionally, there is no truncated or reduced organization of radial glial cells observed in the *Manf* $^{-/-}$ cortex. Since previous studies have demonstrated that neuronal migration and neurite outgrowth are correlated and are dependent on the same molecular mechanisms (Yamasaki et al., 2011; Lee et al., 2012), we assumed that loss of MANF, impairing neurite outgrowth, also caused delayed cortical neuron migration in the developing cortex.

To further elucidate the mechanistic action of MANF in cell migration, we tried to manipulate the level of MANF in the NPCs by isolating SVZ explants from conventional knockout mice lacking MANF mRNA, and by supplementing with exogenous MANF or overexpression of endogenous MANF in DCX^{+} cells. SVZ cells lacking MANF showed a shorter distance of migration as compared with WT cells. Also, we found that administration of MANF could induce neural/glial differentiation accompanied by the morphological change of a bipolar shape as well as promotion of cell migration out of SVZ explants. These data again suggest a critical role of MANF in the differentiation of NSC/NPCs and development of neurite-like and glial processes, and subsequently the migration of SVZ cells. The results of deletion of MANF in NPC cultures imply that MANF deficiency-induced activated UPR signals could be implicated in neurite

outgrowth and indirectly interfere with the process of neuronal migration, although exogenous MANF administration in NPC cultures did not alter the UPR signals compared to the naïve control. Accordingly, the downstream pathways of MANF might be not only in UPR regulation, and would need to be further investigated.

6.6 Possible mechanisms of MANF´s action

To date, MANF has been found to calcium-dependently interact with the ER chaperon GRP78, but is released from GRP78 and secreted upon ER stress which results from depletion of ER calcium. However, there is no direct evidence that MANF, like GRP78, is involved with ER transmembrane receptors to regulate the UPR. Additionally, the signaling pathways for MANF have remained largely unclear, though prior studies have shown that administration of an extremely high dose of recombinant MANF increased PKC phosphorylation in PC12 cells in a time-dependent manner (Yang et al., 2014a). PKC has multiple effects, and its activity modulates many downstream molecular pathways (Corbit et al., 2000), such as MAPK (Corbit et al., 1999). In this work, we first showed that intra- and extracellular administration of MANF has the potential to enhance cell migration from SVZ explants. Moreover, the MANF-overexpressed DCX-positive cells form chain-like structures extending outside of SVZ explants, suggesting that MANF may induce neuroblasts to exhibit migratory morphology. This finding led us to hypothesize that MANF may regulate intracellular signals to exert its effect on cell migration. While overexpressed MANF does not affect the levels of phosphorylation of AKT and the downstream ribosomal protein S6, it triggers phosphorylation of STAT3 and ERK1/2 activation in cells migrating out of SVZ explants. STAT3, a classic transcription regulator, was first discovered as a key mediator of cytokine-induced inflammation and immunity (Yu et al., 2009). Later studies found that STAT3 regulates a much wider range of biological processes, including determining the fate of NSCs. GFAP expression during glial differentiation from NSCs is dependent on the activation of STAT3, especially on the phosphorylation at Tyr705 of STAT3 (Xia et al., 2002). Moreover, activation of STAT3 is crucial for neuronal differentiation because cytoplasmic STAT3 can modulate the microtubule network by antagonizing the microtubule destabilization activity of stathmin (Ng et al., 2006). Furthermore, a number

of studies have shown that serine-phosphorylated STAT3 is localized to the mitochondria instead of the nucleus (Islam et al., 2009; Zhou and Too, 2013), which is involved in NGF-induced neurite outgrowth (Zhou and Too, 2011). In this study, administration of exogenous rhMANF to neurosphere cultures and overexpression of endogenous hMANF in SVZ explants induced activation of STAT3, implying that MANF can regulate the STAT3 pathway to promote NSC differentiation and progenitor cell migration. Recent evidence suggests there may be an important interaction between the UPR receptors and STAT3 signaling. For example, STAT3-activating cytokines, such as IL6, increase the expression of GRP78, a key regulator of UPR activation, but does not induce ER stress (Vollmer et al., 2010). Also, in astrocyte cultures, STAT3 is activated through a novel PERK-JAK1 pathways to regulate ER-induced inflammation (Meares et al., 2014). Does MANF induce activation of STAT3 signaling in NSC/NPCs through interacting with ER transmembrane receptors to affect the JAK-STAT3 pathway or by direct phosphorylation of JAK1? Additional studies will be required to address these questions. Although the overexpression of MANF also caused ERK1/2 phosphorylation in SVZ explants, we do not know whether this activation was directly induced by MANF or STAT3-related downstream targets. Since STAT3 upregulates gene expressions of growth factors, such as VEGF or HIF1α in many cell types (Sakata et al., 2012), we assumed the ability of MANF to induce STAT3 activation not only promotes NSC differentiation, but also increases the levels of these growth factors, which mediate the ERK1/2 pathway to exert the stimulatory effect on the cell migration. Further studies will be required to identify putative MANF receptors in NPCs and the specific contribution of MANF-activated STAT3 to exert these effects.

6.7 The effect of post-stroke MANF and GDNF on neurogenesis and functional recovery

Our better understanding of MANF's roles in the biological properties of NSCs/NPCs encouraged us to utilize the beneficial effect of MANF on these cells for optimizing endogenous stem cell-based therapies to repair the damaged CNS. In order to identify the adult NPC behavior underlying the effect of MANF treatment, we turned to an unbiased approach of comparing MANF with GDNF treatment after cortical stroke. GDNF

increased cell proliferation and the number of neuroblasts in the SVZ, but not at the infarct boundary on day 14 after stroke, which is not similar to a previous finding that GDNF administration enhanced recruitment of neuroblasts in ischemic lesions (Kobayashi et al., 2006). There are several possible explanations for this discrepancy. First, we performed MCAO by ligation of the right MCA and bilateral common carotid arteries for 90 min, which produces the ischemic lesion mainly in the neocortex without compromising the striatum. On the other hand, Kobayashi and coworkers used the intraluminal filament technique to produce a larger infarct, including the striatum and neocortex. This suggests that increased cell proliferation and neuroblast numbers could have been caused by more extensive damage (Kobayashi et al., 2006). Second, the infusion sites of GDNF were different. In our study, GDNF was infused into the lateral ventricle, whereas Kobayashi and coworkers infused GDNF intrastriatally so that GDNF diffused throughout the striatum, including the SVZ. Therefore, recruitment of more neuroblasts to the infarcted striatum may result from a chemoattractant effect or secondarily due to an increase in the number of neuroblasts in the SVZ (Kobayashi et al., 2006). Previous studies have shown that MANF and GDNF have different diffusion and transport profiles after intracerebral injection. Compared to GDNF, the volume of distribution of MANF is significantly larger and more MANF is transported to the frontal cortex after intrastriatal injection (Voutilainen et al., 2009). Since distribution capacity is related to efficacy of neurotrophic factors, the lower benefits of GDNF and NRTN, with their limited tissue distribution, have been shown in recent clinical trials in neurodegenerative disease. Therefore, it could be hypothesized that the increased migration of neuroblasts from the SVZ toward the infarct area of the neocortex seen here is attributed to a direct effect of MANF on neuroblast activity and a better distribution or more efficient transportation of MANF in the cortex. In particular, MANF did not affect proliferation of SVZ cells after stroke, which may be of importance for possible clinical use of MANF because almost all growth factors could induce dysplasia from uncontrolled cell proliferation.

To determine the effect of long-term MANF treatment on forebrain neurogenesis in the stroke model, we used local infusion of MANF to analyze DCX^+ cells in the infarct area

and compared it with GDNF. Similar to GDNF treatment, local infusion of MANF also increased the number of DCX^+ cells in the infarcted cortex. This likely reflects an environment that is more conductive to maintenance of neuroblasts by promoting cell survival rather than enhanced migration toward this region. Although local differences in microenviornments between brain regions are not surprising, the actual source of difference that would account for this change in neuroblast maintenance is unclear.

In addition, MANF and GDNF did not significantly increase the number of newly-generated neurons in the peri-infarct zone, implying that almost all migrating NPCs had not yet differentiated to mature neurons on day 24 after stroke. In our study, the infusion of MANF or GDNF continued for up to 16 days, similar to the temporal pattern of ischemic-induced neurogenesis and neuroblast migration described previously. As levels of MANF or GDNF decreased due to withdrawal of supplementation, migrating neuroblasts in the injured cortex might halt or fail to differentiate into mature neurons. Finally, MANF treatment, but not GDNF, was shown to induce behavioral improvement after stroke. Since post-stroke inflammation has been implicated in MANF treatment (Tobin et al., 2014), it is possible that the effects of MANF on behavioral improvement are mediated by immune modulation (Chen et al., 2015a; Stratoulias and Heino, 2015). Indeed, recent reports have suggested that MANF has an immunomodulatory function to improve the success of cell-replacement regenerative therapies in the retina (Neves et al., 2016). Further studies are needed to determine the roles of MANF in mediating inflammation and increasing recruitment of NPCs in the lesioned cortex to develop better therapeutic strategies for brain injury.

6.8 The therapeutic application of MANF for stroke: challenges and prospects

Although MANF was discovered in 2003, our knowledge about the basic biology, mode of action and therapeutic potential of this unique neurotrophic factor is still limited (Lindahl et al., 2017). The major challenge is to understand the mechanism of action of this protein. Compared to other NTFs this is very challenging since MANF acts inside the cell and most obviously also as an extracellular protein (Glembotski et al., 2012). When MANF was applied as an extracellular protein or delivered by viral vector, it had

neuroprotective effects in rodent models of cerebral ischemia (Airavaara et al., 2009; Airavaara et al., 2010). These promising results raise hopes that MANF can be developed as a novel drug for treatment of ischemic stroke, but several challenges still remain. So far, all neuroprotective agents have failed to show any benefits in humans (Wu, 2005; Uzun et al., 2010). Although there are multiple underlying reasons for the failure, the BBB is an important issue and will continue to be a hurdle in the development of new therapies for acute ischemic stroke. The BBB is a natural barrier that keeps large molecules from entering the brain (Menzies et al., 1993). Even though cerebral ischemia causes disruption of the BBB, it occurs only after several hours of the onset. Since the therapeutic time window for most neuroprotective agents is less than 6 hours, all large molecule agents cannot enter the brain from the blood stream to achieve an effecitivelevel; and this is especially true for neurotrophins, which are endogenously large molecule peptides or proteins (Hefti, 1997). Thus, identifying new strategies that can be used beyond the current time window and regulate post-ischemia inflammation or enhance neurorepair are critically needed. Various research studies as well as clinical trials have been conducted to examine the effect of various anti-inflammatory treatments after ischemic stroke. While these studies offer promising avenues for anti-inflammation in acute ischemic stroke, the fact that blocking post-ischemic inflammation could also eliminate any beneficial effects of inflammation cannot be overlooked (Tobin et al., 2014). For example, chronic inflammation induced by status epilepticus was implicated in increasing the number of mature neurons, the majority of which replaced dead granule cells in the granular cell layer of dentate gyrus (Morrens et al., 2012). In contrast, there is substantial evidence to show that inflammation impairs neurogenesis after injury via increased activation of $ED1^{+}$ microglia and via secretion of numerous proinflammatory cytokines, including TNF-α, and interferon-γ (Ekdahl et al., 2003; Monje et al., 2003). The link between the immune response that occurs after ischemic stroke, and neurogenesis or subsequent functional recovery has not been well established. The dual roles of microglia after stroke are becoming increasingly apparent, and it would appear that the microglial response has both beneficial and detrimental consequences for neurogenesis (Ekdahl et al., 2009).

As discussed in previous sections, MANF has been shown to have immunomodulation effects to improve the success of cell-replacement regenerative therapies in the mouse model of degenerative retina (Neves et al., 2016). This might result in a faster clearance of dead cells to improve a microenvironment. Furthermore, in my present study, MANF is demonstrated to be indispensable for neurite outgrowth and neuronal migration during cortical development. Application of exogenous MANF can promote NSC differentiation *in vitro* as well as NPC migration toward the infarct boundary. Taken together, these findings suggest that MANF could serve as a potential pharmacological agent to boost functional recovery afterf ischemic injury via regulating post-ischemia inflammation and enhancing neuroregeneration. Further studies should be done to explore the molecular mechanisms involved in MANF's regulation of these processes, which may lead to the development of more efficient therapeutic approaches for stroke patients. Also, it is anticipated that combination therapies using MANF and others groups of neurotrophic factors would have additive or synergistic effects in the future treatment of ischemic stroke.

7 CONCLUSIONS

This thesis aimed to elucidate the role of MANF in the developing cortex and to investigate the effects of neurotrophic factors on NSC/NPC survival, proliferation, differentiation, and migration under physiological or pathological conditions. Based on the findings presented in this thesis, the following conclusions can be drawn:

- MANF is expressed in NSCs/NPCs, but loss of MANF has no effects on proliferation or survival of NSCs/NPCs.
- It is indispensable for neurite outgrowth and regulates UPR signals during neuronal differentiation.
- MANF is a major factor regulating cortical development *in vivo* and its loss results in delayed neuron subtype determination, decreased neurite extension, and slower neuronal migration.
- Administration of exogenous MANF triggers NSC differentiation accompanied by increased neuronal- as well as glial-related cytoskeletal proteins, and facilitates cell migration from SVZ explants.
- Overexpression of MANF induces the phosphorylation of STAT3 and ERK1/2 during SVZ cell migration.
- MANF promotes the migration of neuroblasts toward the ischemic cortex and further contributes to their recruitment within infarcted neocortex.
- Endogenous MANF has a neuroprotective effect in a mouse model of permanent cortical ischemia.
- Although GDNF increased proliferation of SVZ cells after stroke, it did not significantly promote the migration of neuroblasts towards ischemic cortex or improve functional recovery.

- Focal delivery of AAV2-BDNF to the non-lesioned side of the SVZ increases migration of NPCs in SVZ and improves behavioral function in stroke animals.

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